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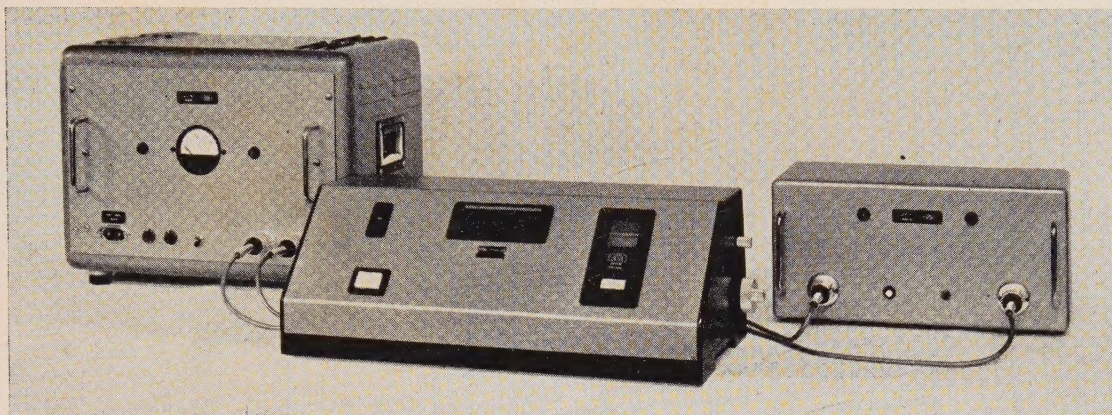


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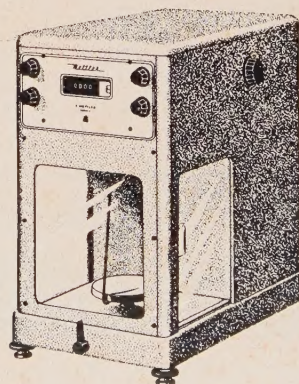
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...und weitere  
in Vorbereitung



## Über eisenhaltige Wachstumsfaktoren, die Sideramine, und ihre Antagonisten, die eisenhaltigen Antibiotika Sideromycine<sup>1</sup>

Von H. BICKEL<sup>2</sup>, E. GÄUMANN<sup>3</sup>, W. KELLER-SCHIERLEIN<sup>4</sup>, V. PRELOG<sup>4</sup>, E. VISCHER<sup>2</sup>,  
A. WETTSTEIN<sup>2</sup> und H. ZÄHNER<sup>3</sup>

1. *Ferrimycine und Ferrioxamine.* Im Verlaufe unserer Untersuchungen über antibiotikabildende Actinomyceten konnten wir braunrote, antibiotisch hochwirksame Stoffe isolieren, deren charakteristisches Merkmal es ist, dass sie komplex gebundenes Eisen enthalten. Sie sind sowohl *in vitro* als auch *in vivo* besonders gegen gram-positive Mikroorganismen stark wirksam. Die von uns am eingehendsten untersuchten Antibiotika aus *Streptomyces griseoflavus* (Krinsky) Waksman et Henrici (Stamm ETH 9578) nennen wir *Ferrimycine*.

Weitere Untersuchungen von Stoffwechselprodukten der Actinomyceten, bei welchen die für Ferrimycine entwickelten Isolierungsmethoden verwendet wurden, führten zu anscheinend verwandten, rotbraunen eisenhaltigen Verbindungen, die zwar antibiotisch unwirksam waren, sich jedoch sowohl *in vitro* als auch *in vivo* als hochwirksame Antagonisten der Ferrimycine entpuppten. Diese Stoffe nennen wir *Ferrioxamine*. Das eingehendere Studium des Antagonismus zwischen Ferrimycinen und Ferrioxaminen zeigte, dass die letzteren Wuchsstoffe für Mikroorganismen sind und dass der Antagonismus auf einer Hemmung der Wuchsstoffwirkung beruht.

2. *Der Antagonismus-Test.* Der Antagonismus zwischen Ferrioxaminen und Ferrimycinen erlaubte für beide Verbindungsgruppen mikrobiologische Tests auszuarbeiten. Mit diesen lassen sich die genannten Wachstumsfaktoren und Antibiotika und – wie wir später zeigen werden – auch weitere verwandte Stoffe noch in starker Verdünnung ( $< 10^{-7}$ ) nachweisen und bestimmen, was ihre Auffindung und Isolierung bedeutend erleichterte<sup>5</sup>.

Eine Ausführungsform der Tests, welche die antagonistische Wirkung der Ferrioxamine und der Ferrimycine veranschaulicht, ist in Abbildung 1 dargestellt. Ein Filterpapierstreifen wird mit einer verdünnten Ferrimycin-Lösung, ein zweiter mit Ferrioxamin-

Lösung getränkt, und beide werden in gekreuzter Lage auf Antibiotika-Testplatten gelegt. Im Hemmhof, welcher sich um den Streifen mit dem Antibiotikum bildet, entsteht an der Kreuzung der beiden Streifen eine keilförmige Einschnürung, deren Form und Dimensionen unter Standardbedingungen zur quantitativen Bestimmung verwendet werden können.

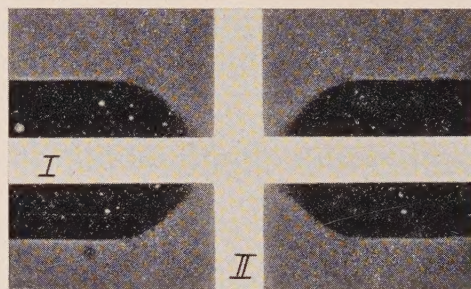


Abb. 1. Antagonismus zwischen Ferrioxaminen (Streifen II) und Ferrimycinen (Streifen I) (natürliche Grösse) Testorganismus: *Bacillus subtilis*

3. *Sideromycine.* Mit Hilfe dieses Testes konnten wir bisher ungefähr 50 Actinomycetenstämme als Produzenten von Antibiotika erkennen, deren Wirkung durch Ferrioxamine aufgehoben wird. Diese Antibiotika sind lediglich in stark polaren Lösungsmitteln, wie zum Beispiel Wasser, Dimethylformamid, Glykol und Methylcellosolve, und einzelne noch in Methanol löslich. Eine besondere Eigenschaft ist ihre gute Löslichkeit in Phenol oder in Gemischen von Phenol und Lipoidlösungsmitteln. Sie lassen sich durch Adsorption, Fällung,

<sup>1</sup> 22. Mitteilung, Stoffwechselprodukte von Actinomyceten; 21. Mitteilung: Helv. chim. Acta 43, im Druck (1960).

<sup>2</sup> Forschungslaboratorien der CIBA Aktiengesellschaft, Basel, Pharmazeutische Abteilung.

<sup>3</sup> Institut für spezielle Botanik der ETH, Zürich.

<sup>4</sup> Organisch-chemisches Laboratorium der ETH, Zürich.

<sup>5</sup> H. ZÄHNER, R. HÜTTER und E. BACHMANN, Arch. Mikrobiol., im Druck (1960).



Verteilung, Chromatographie und Elektrophorese von ihren Begleitstoffen trennen und durch Papierchromatographie und Papierelektrophorese charakterisieren.

Unter den bekannten Stoffwechselprodukten von Actinomyceten befinden sich bereits einige antibiotisch wirksame Substanzen, die durch ihren Gehalt an Eisen auffallen. Der erste Vertreter dieser Gruppe, das gegen gram-positive und -negative Keime wirksame Grisein, wurde schon 1947 von WAKSMAN *et al.*<sup>6</sup> in den Kulturfiltraten eines Stammes von *Streptomyces griseus* Waksman et Henrici gefunden und von KUEHL *et al.*<sup>7</sup> in hochgereinigter Form als rotes Pulver isoliert. GAUSE und BRAZHNKOVA<sup>8</sup> fanden 1951 ein weiteres eisenhaltiges Antibiotikum, das Albomycin, das von einer als *Actinomyces subtropicus* Kudrina et Kochetkova<sup>9</sup> bezeichneten Streptomycetenart gebildet wird. Es zeigte gemäss STAPLEY und ORMOND<sup>10</sup> sehr ähnliche physikalisch-chemische und mikrobiologische Eigenschaften wie Grisein. Das von BRAZHNKOVA *et al.*<sup>11</sup> vorerst als einheitliche Hauptkomponente des Albomycincomplexes aufgefasste  $\delta$ -Albomycin wurde neuerdings von MIKEŠ und ŠORM<sup>12</sup> in 2 Komponenten  $\delta_1$  und  $\delta_2$  aufgetrennt. Die Anreicherung einer weiteren Grisein-ähnlichen Substanz, des Antibiotikums A 1787, wurde von THRUM<sup>13</sup> untersucht, und schliesslich sind kürzlich von SENSI und TIMBAL<sup>14</sup> antibiotisch wirksame Substanzen, die mit Grisein gekreuzte Resistenz zeigen, aus Kulturfiltraten von zwei nicht klassifizierten Streptomyces-Stämmen L. A. 5352 und L. A. 5937 erhalten worden.

Diese Stoffe zeigen die gleiche antagonistische Wirkung gegen die Ferrioxamine wie die Ferrimycine. Wir schlagen für solche eisenhaltige Antibiotika – sowohl für die von uns gefundenen als auch für die früher beschriebenen – den Oberbegriff *Sideromycine* vor. Die anderen bekannten Antibiotika, die wir in grosser Zahl geprüft haben, werden von Ferrioxaminen nicht antagonistisch beeinflusst. Die Sideromycine sind weiter charakterisiert durch eine hohe Resistenz-Bildungsrate und dadurch, dass sie untereinander gekreuzte Resistenz zeigen. Die drei Eigenschaften – der Antagonismus, die rasche Resistenzbildung und gekreuzte Resistenz – stehen wahrscheinlich in ursächlichem Zusammenhang.

Die Sideromycine lassen sich gemäss ihrem Wirkungsspektrum und anhand ihres papierchromatographischen Verhaltens vorderhand in 3 Gruppen einteilen. Gruppe 1 umfasst diejenigen mit breitem antibiotischem Spektrum, darunter Grisein und Albomycin<sup>15</sup>. Die hemmende Wirkung dieser Sideromycine wird überraschenderweise durch Ferrioxamine nur bei gram-positiven Mikroorganismen aufgehoben, dagegen nicht bei gram-negativen. Sideromycine der Gruppen 2 und 3 sind vorwiegend gegen gram-positive Keime wirksam. Sie unterscheiden sich deutlich durch ihr papierchromatographisches Verhalten.

Für die Einteilung der Sideromycine hat sich Papierchromatographie im System Butanol-Eisessig-Wasser (4:1:5) am besten bewährt (vgl. auch STAPLEY und ORMOND<sup>10</sup>). Das Verhalten der drei Gruppen in diesem Fliessmittel ist schematisch in den Abbildungen 2a und 2b dargestellt. Die aktiven Substanzen wurden bioautographisch mit *Staphylococcus aureus* und *Escherichia coli* nachgewiesen. Abbildung 2a zeigt das Papierchromatogramm einiger Vertreter der Gruppe 1 bei langer Laufzeit des Fliessmittels. Vergleichsweise wurde ein Antibiotikum der Gruppe 2 (A 22765) und ein Vertreter der Gruppe 3 (Ferrimycin) mitchromatographiert. In Abbildung 2b sind – im gleichen System aber bei kurzer Laufzeit – einige Vertreter der 2. und 3. Gruppe mit einem Repräsentanten der Grisein-Gruppe (Albomycin) aufgeführt. Ähnliche Chromato-

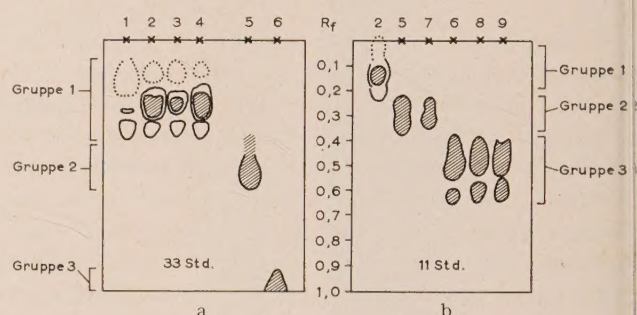


Abb. 2. Papierchromatogramm mit Sideromycinen. System Butanol-Eisessig-Wasser (4:1:5), Bioautogramme mit *Escherichia coli* und *Staphylococcus aureus*

- 1 Grisein (Merck & Co., 40000 E/mg).
- 2 Albomycin (500000 E/mg).
- 3 A 1787 (H. THRUM).
- 4 Antibiotikum aus *Streptomyces griseus*, ETH 10073
- 5 Antibiotikum aus *Streptomyces aureofaciens*, ETH 22765
- 6 Ferrimycine aus *Streptomyces griseoflavus*, ETH 9578
- 7 Antibiotikum aus *Streptomyces aureofaciens*, ETH 22931
- 8 Ferrimycine aus *Streptomyces galilaeus*, ETH 18822.
- 9 Ferrimycine aus *Streptomyces lavendulae*, ETH 21510
- Hemmzonen auf *Staphylococcus aureus*
- starke, ○ schwache Hemmzonen auf *Escherichia coli*.

<sup>6</sup> D. M. REYNOLDS, A. SCHATZ und S. A. WAKSMAN, Proc. Soc. exp. Biol. Med., N. Y. 64, 50 (1947). – D. M. REYNOLDS und S. A. WAKSMAN, J. Bact. 55, 739 (1948).

<sup>7</sup> F. A. KUEHL, M. N. BISHOP, L. CHAIET und K. FOLKERS, J. Amer. chem. Soc. 73, 1770 (1951).

<sup>8</sup> G. F. GAUSE und M. G. BRAZHNKOVA, Nov. Med. (Moskau) 23, 3 (1951). – G. F. GAUSE, Brit. med. J. 1955, 1177.

<sup>9</sup> E. S. KUDRINA und G. V. KOCHETKOVA, Antibiotiki (Moskau) 3, 63 (1958).

<sup>10</sup> E. O. STAPLEY und R. E. ORMOND, Science 125, 587 (1957).

<sup>11</sup> M. G. BRAZHNKOVA, O. MIKEŠ und N. N. LOMAKINA, Biochimija (Moskau) 22, 111 (1957).

<sup>12</sup> O. MIKEŠ und F. ŠORM, Symposium on Antibiotics, Prag (1959), 30.

<sup>13</sup> H. THRUM, Naturwiss. 44, 561 (1957).

<sup>14</sup> P. SENSI und M. T. TIMBAL, Antibiotics & Chemotherapy 9, 160 (1959).

<sup>15</sup> Wir danken Dr. K. FOLKERS, Merck & Co., Inc., Rahway, N. J., USA., und Dr. H. THRUM, Jena, für die Überlassung von Vergleichsproben.



gramme ergeben sich in einer Reihe weiterer Lösungsmittelsysteme. Wie ersichtlich, enthielten die untersuchten Antibiotikapräparate mehrere aktive Komponenten, die sich durch Verwendung eines für die betreffende Gruppe speziell geeigneten Lösungsmittelsystems chromatographisch noch besser auftrennen liessen. Sämtliche Komponenten einer Gruppe sind verschieden von sämtlichen Komponenten der anderen Gruppen. Dagegen scheinen, gemäss den bisherigen papierchromatographischen Versuchen, die der gleichen Gruppe zugezählten Antibiotika jeweils die gleichen aktiven Komponenten, aber in verschiedenen relativen Mengenverhältnissen zu enthalten<sup>16</sup>.

4. *Sideramine*. Die Ferrioxamine besitzen ausgesprochene Wuchsstoffeigenschaften für eine Reihe von Mikroorganismen, zum Beispiel für *Bacillus subtilis*, *Staphylococcus aureus*, *Ustilago sphaerogena* Burill ex Ellis et Everh. und *Chlamydomonas eugametos* Moewus.

Andere, von den Ferrioxaminen verschiedene, eisenhaltige oder eisenbindende Verbindungen mit Wuchsstoffcharakter für Mikroorganismen sind bereits beschrieben worden. NEILANDS *et al.*<sup>17</sup> isolierten aus *Ustilago sphaerogena* Burill ex Ellis et Everh. das Ferrichrom und das Ferrichrom A. Diese zeigten ebenso wie das Coprogen, das HESSELTINE *et al.*<sup>18</sup> aus einer *Penicillium*-Species isolierten, Vitamincharakter für *Pilobolus kleinii* van Tieghem. LOCHHEAD *et al.*<sup>19</sup> erhielten aus Kulturfiltraten von *Arthrobacter pascens* Lochhead et Burton einen als Terregens-Faktor bezeichneten, für *Arthrobacter terregens* Lochhead et Burton essenziellen, Eisenkomplex bildenden Wirkstoff, der durch Ferrichrom oder Coprogen ersetzt werden konnte.

Es war naheliegend, auch diese Verbindungen auf Antisideromycin-Wirksamkeit zu prüfen<sup>20</sup>. Ferrichrom zeigte in unseren Versuchen mit *Bacillus subtilis* eine dem Ferrioxamin B, dem bis jetzt am eingehendsten untersuchten Ferrioxamin, ebenbürtige antagonistische Wirkung. Dagegen war Ferrichrom A in Konzentration bis zu 100  $\gamma$ /ml unwirksam und konnte in höheren Konzentrationen aus Substanzmangel nicht geprüft werden. Der Terregens-Faktor, der uns nur in Form eines rohen Kulturfiltrates von *Arthrobacter pascens* zur Verfügung stand, zeigte deutliche antagonistische Wirkung gegen Ferrimycin und umgekehrt, konnte Ferrioxamin die Wuchsstoff-Wirkung des Terregens-Faktors für *Arthrobacter terregens* und *Arthrobacter flavescens* Lochhead übernehmen. Von Coprogen standen uns keine Vergleichsproben oder Produzentenstämme zur Verfügung. Es ist jedoch wahrscheinlich, dass auch Coprogen Antisideromycin-Wirksamkeit besitzt, da es sich in anderen biologischen Wirkungen durch Ferrichrom und den Terregens-Faktor ersetzen lässt<sup>21</sup>.

Die ganze Gruppe dieser Wuchsstoffe bzw. Vitamine für Mikroorganismen möchten wir unter der Bezeichnung *Sideramine* zusammenfassen. Sie sind chemisch als Eisenkomplexe (oder Eisenkomplexbildner<sup>22</sup>) und

biologisch als Antagonisten der Sideromycine gekennzeichnet. Es handelt sich um ein seltenes Beispiel von Antibiotika-Antagonisten mikrobieller Herkunft. Sie entstehen sogar oft in Kulturen des gleichen Mikroorganismus, der das Antibiotikum produziert (vgl. S. 133). Für die Abklärung ihrer Bedeutung im mikrobiologischen, pflanzlichen und tierischen Stoffwechsel sind weitere Untersuchungen im Gange.

5. *Siderochrome*. Alle diese verwandten eisenhaltigen Naturstoffe, welche die typische Eisenkomplex-Absorption bei 420–440 m $\mu$  zeigen, und zwar die biologisch aktiven wie diejenigen, deren biologische Funktion noch nicht evident ist, bezeichnen wir mit dem Namen *Siderochrome*. In der folgenden Übersicht sind die wichtigsten alten und neuen Siderochrome nochmals zusammengestellt.

| Siderochrome      |                     |                                      |
|-------------------|---------------------|--------------------------------------|
| Wachstumsfaktoren | Antibiotika         | Biologisch unabgeklärte Siderochrome |
| <i>Sideramine</i> | <i>Sideromycine</i> |                                      |
| Ferrioxamine      | Ferrimycine         |                                      |
| Ferrichrome       | Griseine            |                                      |
| Coprogen          | Albomycine          |                                      |
| Terregens-Faktor  | usw.                |                                      |

6. *Isolierung und Eigenschaften der Ferrimycine*<sup>24</sup>. Die Isolierung der Ferrimycine gestaltete sich vor allem deshalb schwierig, weil diese Antibiotika in den Kulturfiltraten in relativ geringer Konzentration und neben grossen Mengen von Substanzen aus der Nährlösung sowie inaktiven Fermentationsprodukten mit zum Teil ähnlichen physikalisch-chemischen Eigen-

<sup>16</sup> Diese Feststellung bedarf in Anbetracht der im Verlaufe eingehender Bearbeitung von Grisein<sup>7-10</sup>, Albomycin<sup>12</sup> und Ferrimycin<sup>24</sup> festgestellten Komplexheit der Sideromycine einer weiteren experimentellen Bestätigung.

<sup>17</sup> J. B. NEILANDS, J. Amer. chem. Soc. 74, 4846 (1952); J. biol. Chem. 205, 643, 647 (1953); Bact. Rev. 21, 101 (1957). – J. A. GARIBALDI und J. B. NEILANDS, J. Amer. chem. Soc. 77, 2429 (1955).

<sup>18</sup> C. W. HESSELTINE, C. PIDACKS, A. R. WHITEHALL, N. BOHONOS, B. L. HUTCHINGS und J. H. WILLIAMS, J. Amer. chem. Soc. 74, 1362 (1952). – C. W. HESSELTINE, A. R. WHITEHILL, C. PIDACKS, M. T. HAGEN, N. BOHONOS, B. L. HUTCHINGS und J. H. WILLIAMS, Mycologia 45, 7 (1953). – C. PIDACKS, A. R. WHITEHILL, L. PRUESS, C. W. HESSELTINE, B. L. HUTCHINGS, N. BOHONOS und J. H. WILLIAMS, J. Amer. chem. Soc. 75, 6064 (1953).

<sup>19</sup> A. G. LOCHHEAD, M. O. BURTON und R. H. THEXTON, Nature 170, 282 (1952). – A. G. LOCHHEAD und M. O. BURTON, Canad. J. Botany 31, 7 (1953). – M. O. BURTON, F. J. SOWDEN und A. G. LOCHHEAD, Canad. J. Biochem. Physiol. 32, 400 (1954).

<sup>20</sup> Wir möchten in diesem Zusammenhang Prof. J. B. NEILANDS, Berkeley, California, für die Überlassung von Präparaten und Prof. A. G. LOCHHEAD, Ottawa, Kanada, für die Zusendung von Kulturen danken.

<sup>21</sup> Vgl. auch A. L. DEMAIN und D. HENDLIN, J. gen. Microbiol. 21, 72 (1959).

<sup>22</sup> Es konnte noch nicht abgeklärt werden, ob das Mycobactin aus *Mycobacterium phlei*, ein Wuchsstoff für *Mycobacterium johnnei*<sup>23</sup>, zu den Sideraminen zu zählen ist. Mycobactin enthält kein Eisen, bildet aber als Hydroxamsäure leicht Eisenkomplexe.

<sup>23</sup> J. FRANCIS, H. M. MACTURK, J. MADINAVEITIA und G. A. SNOW, Biochem. J. 55, 596 (1953). – G. A. SNOW, J. chem. Soc. 1954, 2588, 4080.

<sup>24</sup> H. BICKEL *et al.*, Helv. chim. Acta, in Vorbereitung.



schaften anfielen. Die Reindarstellung über die relativ grosse Anreicherungsspanne wurde zudem dadurch erschwert, dass die Wirkstoffe Gemische engverwandter, relativ hochmolekularer und empfindlicher Verbindungen darstellten.

Ferrimycine liessen sich aus den Kulturfiltraten des Stammes *Streptomyces griseoflavus* (Krainsky) Waksman et Henrici ETH 9578<sup>25</sup>) an Amberlite IRC 50 adsorbieren, mit wässrig methanolischer Salzsäure eluieren und durch anschliessende Fällungs- und Extraktionsoperationen mit Phenol und Phenol-Chloroform-Gemischen in bereits stark angereicherter Form als Gemisch von Ferrimycin A und dem in untergeordneter und variierender Menge auftretenden, noch nicht vollständig charakterisierten Ferrimycin B (vgl. Abb. 2b) gewinnen. Eine weitere Anreicherung von A unter Abtrennung von B erfolgte durch Craigsche Gegenstromverteilung zwischen Benzylalkohol-Butanol (2:1) und Kochsalz enthaltender verdünnter Salzsäure. Durch anschliessende Chromatographie an Dowex 50-X2 oder Gegenstromelektrophorese in verdünnter Essigsäure erhielten wir Ferrimycin A in Form eines dunkelbraunroten, wasser- und methanollöslichen Pulvers. *Es erwies sich im experimentellen Tierversuch bei vergleichbarer therapeutischer Breite 10–50mal aktiver als Penicillin und ist demnach eines der wirksamsten Antibiotika gegenüber gram-positiven Bakterien*<sup>26</sup>. Seine chemischen und physikalischen Eigenschaften sind in der Tabelle denjenigen von Grisein A und Albomycin – als den am besten charakterisierten Breitspektrum-Sideromycinen – gegenübergestellt (vgl. auch Abb. 3). Ein Vergleich dieser Daten bestätigt die anfänglich nur durch biologische Merkmale angezeigte Verwandtschaft. Ferri-

mycin A, das in den meisten Lösungsmittelgemischen papierchromatographisch einheitlich erscheint, liess sich bei langdauernder Chromatographie an Cellulose-säulen unter Verwendung von Lösungsmittelsystemen, in denen es einen sehr kleinen  $R_f$ -Wert aufweist, in zwei Komponenten,  $A_1$  und  $A_2$ , auftrennen, die wir bis anhin nur durch den verschiedenen  $R_f$ -Wert in diesen Systemen unterscheiden konnten.

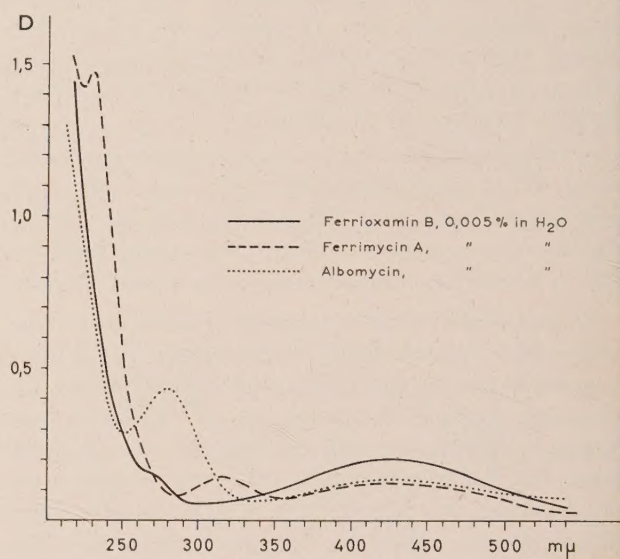


Abb. 3. UV.-Spektren von Ferrioxamin B, Ferrimycin A, Albomycin (500 000 E/mg)

<sup>25</sup> Über die Fermentierung von Stamm ETH 9578 siehe P. REUSSER *et al.*, in Vorbereitung.

<sup>26</sup> L. NEIPP *et al.*, in Vorbereitung.

| Substanz                     | Analysenwerte (%) |      |       |      |      | Mol.-Gew.              | $pK_{MCS}^*$ <sup>d</sup> | Absorption        |                        | Nachgewiesene Hydrolysenprodukte                                                                                                                                                                                                   | e      |
|------------------------------|-------------------|------|-------|------|------|------------------------|---------------------------|-------------------|------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------|
|                              | C                 | H    | N     | Cl   | Fe   |                        |                           | $\lambda_{max}$   | $E_{1\%}^{1\text{cm}}$ |                                                                                                                                                                                                                                    |        |
| Ferrimycin A <sup>24</sup>   | 48,65             | 7,09 | 12,95 | 6,10 | 4,56 | 1106 <sup>a</sup>      | 4,18; 7,88                | 228<br>319<br>425 | 282<br>28,2<br>22,6    | NH <sub>3</sub> , Bernsteinsäure, 1-Amino-5-hydroxylamino-pentan, $\delta$ -Amino-valeriansäure, Cadaverin, krist. Substanz mit $\lambda_{max}$ 227 und 323m $\mu$ , Prolin und nicht identifizierte ninhydrin-positive Substanzen | (0,83) |
| Grisein A <sup>7,10</sup>    | 43,95             | 5,65 | 12,97 |      | 5,14 | 1034 <sup>a</sup>      |                           | 265<br>420        | 108<br>28,9            | Methyluracil, Glutaminsäure                                                                                                                                                                                                        |        |
| Albomycin <sup>8,11,12</sup> |                   |      |       |      | 4,16 | 1270–1346 <sup>b</sup> |                           |                   |                        | Methyluracil, Serin, Ornithin                                                                                                                                                                                                      |        |
| Ferrioxamin B <sup>29</sup>  | 48,04             | 7,41 | 11,21 | 5,25 | 7,67 | 704 <sup>a</sup>       | 9,74                      | 430               | 39,0                   | NH <sub>3</sub> , Bernsteinsäure, 1-Amino-5-hydroxylamino-pentan, Cadaverin, $\delta$ -Aminovaleriansäure, Essigsäure                                                                                                              | (1,2)  |
| Ferrichrom <sup>17</sup>     | 44,02             | 5,90 | 16,55 |      | 7,35 | 725 <sup>c</sup>       |                           | 425               | 39,4                   | NH <sub>3</sub> , Glycin, Ornithin                                                                                                                                                                                                 | 2,89   |
| Ferrichrom A <sup>17</sup>   | 44,75             | 5,80 | 11,18 |      | 5,3  | 1100 <sup>c</sup>      |                           | 440               | 33,8                   | Serin, Glycin, Ornithin                                                                                                                                                                                                            | 3,01   |
| Coprogen <sup>18</sup>       | 50,96             | 6,83 | 10,26 |      | 6,61 |                        |                           | 440               | 36,6                   |                                                                                                                                                                                                                                    |        |

<sup>a</sup> Durch Titration. <sup>b</sup> Gemäss Fe-, SO<sub>4</sub>- und NH<sub>3</sub>-Gehalt. <sup>c</sup> Durch zwei unabhängige Methoden ermittelt. <sup>d</sup> W. SIMON, E. KOVÁTS, H. CH. LOPARD-DIT-JEAN und E. HEILBRONNER, Helv. 37, 1872 (1954). <sup>e</sup> Kolorimetrisch nach CSÁKY<sup>25</sup> bestimmte «Hydroxylamin-Werte» pro Atom Fe. Die in Klammern angegebenen Werte sind unkorrigiert.



7. *Isolierung und Eigenschaften der Ferrioxamine.* Ferrioxamine wurden in den Stoffwechselprodukten einer grösseren Anzahl von Actinomyceten, insbesondere in Stämmen der Arten *Streptomyces griseoflavus* (Krainsky) Waksman et Henrici, *S. pilosus* Ettlinger et al., *S. viridochromogenes* (Krainsky) Waksman et Henrici, *S. olivaceus* (Waksman) Waksman et Henrici, *S. aureofaciens* Duggar, *S. galilaeus* Ettlinger et al., *S. lavendulae* (Waksman et Curtis) Waksman et Henrici, *S. polychromogenes* Hagemann et al., und *S. griseus* Waksman et Henrici gefunden.

Wir haben uns bisher vorwiegend mit der chemischen Bearbeitung des Ferrioxamins aus dem *Streptomyces pilosus*-Stamm ETH 21748 beschäftigt. Gemäss Papierchromatographie enthielten die Kulturfiltrate dieses Stammes 3–5 Ferrioxamine. Die als Ferrioxamin B bezeichnete Hauptkomponente liess sich, entsprechend unserer Erwartung, mit den beim Ferrimycin entwickelten Isolierungsmethoden in Form eines wasserlöslichen, eisenhaltigen, braunroten Pulvers gewinnen. Seine physikalischen und chemischen Eigenschaften sind in der Tabelle und in Abbildung 3 aufgeführt. Die offensichtliche chemische Verwandtschaft des Ferrioxamins B mit Ferrimycin und den Breitspektrum-Sideromycinen wird weiter durch biogenetische Zusammenhänge unterstrichen. Wir konnten nämlich Ferrioxamine neben den antibiotisch wirksamen Stoffen in den Kulturfiltraten der meisten Sideromycinbildner nachweisen, wobei das relative Mengenverhältnis durch die Züchtungsbedingungen beeinflussbar war. Im Falle der Fermentation von *S. griseoflavus* wurden kleine Mengen von Ferrioxamin B neben dem Hauptprodukt Ferrimycin A in annähernd reiner Form isoliert.

In der Tabelle sind die bekannten physikalischen und chemischen Daten der anderen Sideramine im Vergleich mit denjenigen von Ferrioxamin B aufgeführt. Wie diese Daten und besonders die Befunde NEILANDS zeigen, lassen sich die Ferrichrome, Coprogen und der Terregens-Faktor eindeutig voneinander unterscheiden. Die in den Kulturfiltraten von *S. pilosus* Ettlinger et al. ETH 21748 nachweisbaren, bzw. daraus isolierten Ferrioxamine waren gemäss direktem papierchromatographischem Vergleich von Ferrichrom und Ferrichrom A verschieden.

Es sei noch hervorgehoben, dass sowohl das Ferrimycin A, als auch das Ferrioxamin B zwei wesentliche Hydrolyseprodukte, das 1-Amino-5-hydroxylaminopentan und die Bernsteinsäure, liefern, welche hydroxamsäure-artig gebunden sind. Das dreiwertige Eisen liegt im Ferrimycin A und im Ferrioxamin B offenbar als Hydroxamsäure-Chelat vor.

EMERY und NEILANDS<sup>27</sup> zeigten vor kurzem an Hand von spektroskopischen Studien und durch den Nachweis von Hydroxylamin, dass das Eisen enthaltende Zentrum von Ferrichrom und Ferrichrom A

ebenfalls als ein Ferri-tri-hydroxamat-Komplex aufzufassen ist. Auch in Grisein und im Terregens-Faktor konnten sie gebundenes Hydroxylamin nachweisen. Die von uns mit Ferrimycin A und Ferrioxamin B, nach der Methode von CSÁKY<sup>28</sup>, erhaltenen, unkorrigierten Hydroxylamin-Werte sind in der Tabelle mit aufgeführt. Ähnliche Werte fanden wir auch bei Albomycin und Grisein.

8. *Ausblick.* Bei der Wichtigkeit, welche Eisen im biologischen Geschehen spielt, kann man das verbreitete Vorkommen von biologisch so hoch wirksamen eisenhaltigen Verbindungen wie die Sideramine und die Sideromycine kaum überschätzen. In unseren Laboratorien sind umfangreiche Untersuchungen im Gange, um verschiedene biologische, chemische und biochemische Probleme, welche mit der interessanten Gruppe der Siderochrome im Zusammenhang stehen, abzuklären.

Summary

A new group of iron-containing metabolites, with growth stimulating properties for a number of microorganisms, has been isolated from streptomycetes and named *ferrioxamines*. It is proposed to include them, together with some known substances, like ferrichrome, coprogen and the terregens factor, which either contain or bind iron, in a new class of growth factors, the *sideramines*.

The biological property of the sideramines is counteracted by iron-containing antibiotics from streptomycetes, the *sideromycins*. They comprise, besides known products like grisein and albomycin, two new groups of antibiotics, among them the highly potent *ferrimycins*.

<sup>27</sup> T. EMERY und J. B. NEILANDS, Nature 184, 1632 (1959).  
<sup>28</sup> T. Z. CSÁKY, Acta chem. scand. 2, 450 (1948).  
<sup>29</sup> Unveröffentlichte Versuche von G. E. HALL.



# Brèves communications - Kurze Mitteilungen Brevi comunicazioni - Brief Reports

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## The Electron-Induced Dissociation of some Monoterpenoids

The studies previously reported upon certain steroids and triterpenoids have now been extended to some monoterpenoids and their derivatives.

These experiments were generally conducted with an electron beam energy of some 12-15 electron volts, a practice previously described<sup>1</sup> which has the effect of simplifying the mass-spectrum. Comparison with many of the published mass-spectra<sup>2</sup> which have been obtained under more usual conditions show little alteration in the main features of the 'cracking-pattern' and although relative intensities of the fragment ions within a spectrum may change somewhat with the electron beam energy the order of intensities is unaffected.

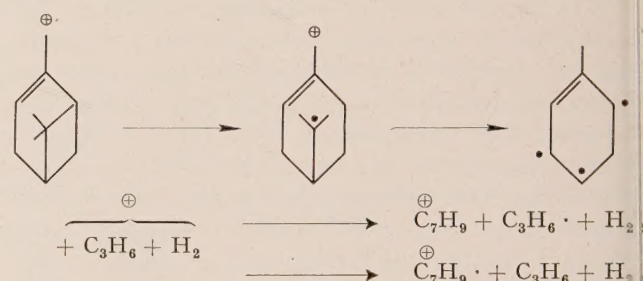
The results are shown in the following Table.

A comparison of the fragmentation patterns of the hydrocarbons shows that the probability of elimination of a methyl group is related to the molecular structure and diminishes along the series  $\Delta^3$  carene, camphene,  $\alpha$ - and  $\gamma$ -fenchene, tricyclene, cyclofenchene, to  $\alpha$ -pinene and dipentene, and camphane. This effect is in agreement with the observations previously discussed<sup>1</sup> that (a) the presence of *gem*-dimethyl groups and (b) the methyl group being allylic to a double bond are two conditions which facilitate group elimination. When both occur together, as in camphene, the elimination is very much more marked than when only one is present. In the absence of both, the loss of the methyl group is not very marked, the exceptional case being  $\Delta^3$  carene. In this compound the parent molecular ion is small under the present experimental conditions the most prominent peak corresponding to a mass of 121. Thus ring strain may be involved in determining the nature of the fragmentation process.

A second feature common to the compounds camphene,  $\alpha$ - and  $\delta$ -fenchene, tricyclene, cyclofenchene, and  $\alpha$ -pinene is that the base peak i.e. the most abundant fragment ion

present corresponds to the loss of 43 mass units. This may be readily correlated with the loss of the bridge structure  $C_3H_6$  and a further hydrogen. Such reactions which involve hydrogen transfer are known to occur with very great facility. It is considered by McLafferty<sup>3</sup> that such re-arrangements, especially those that allow the formation of double-bonds, are favoured over simple bond fissions as they result in the formation of one bond for each bond broken. Moreover, such re-arrangements involve the migration of a hydrogen atom on the  $\beta$ -carbon atom to the ruptured bond. This is supported by all the cases listed by the observation that the largest fragment of low mass obtained has  $M/e = 41$  resulting from the extraction of one hydrogen atom from each fragment with the formation of a hydrogen molecule; a particularly common form of re-arrangement.

A typical fragmentation process may therefore be written



The presence of an allylic system must obviously favour the initial rupture of the  $C_3H_6$  fragment. The alternative mode of break-down which might occur, namely, the migration of a hydrogen atom from the ring to the  $C_3H_6$

<sup>1</sup> R. I. REED, J. chem. Soc. 1958, 3432.

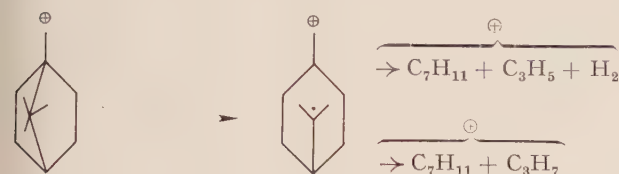
<sup>2</sup> A. P. I. Reports, Project No. 44.

<sup>3</sup> F. W. McLafferty, A.S.T.M.E-14 Committee on Mass Spectrometry, May 1958.

| Compound                | 154 | 138 | 136 | 123 | Mass of Ion |                       |    |        |    |             |    |        |    |     |    |
|-------------------------|-----|-----|-----|-----|-------------|-----------------------|----|--------|----|-------------|----|--------|----|-----|----|
|                         |     |     |     |     | 121         | 95                    | 93 | 81     | 79 | 68          | 57 | 55     | 43 | 41  | 28 |
| $\Delta^3$ Carene . . . | —   | —   | —   | —   | B           |                       |    |        |    |             |    |        |    |     |    |
| Camphene . . .          | —   | —   | W   |     | S           |                       | B  |        |    |             |    |        |    | VS  |    |
| $\alpha$ -Fenchene . .  | —   | —   | M   |     | MS          |                       | B  |        | S  |             |    |        |    | VS  |    |
| $\delta$ -Fenchene . .  | —   | —   | M   |     | MS          |                       | B  |        | S  |             |    |        |    | VS  |    |
| Tricyclene . . .        | —   | —   | M   |     | M           |                       | B  |        |    |             |    |        |    | M   |    |
| Cyclofenchene .         | —   | —   | W   |     | M           |                       | B  |        |    |             |    |        |    | M   |    |
| $\alpha$ -Pinene . . .  | —   | —   | VW  |     | W           |                       | B  |        |    |             |    |        |    | M   |    |
| Dipentene . . .         | —   | —   | W   |     | W           |                       | S  |        |    | B           |    |        |    | S   |    |
| Camphane . . .          | —   | —   | M   | M   |             | VS                    |    | S      |    |             |    | B      | M  | VS  |    |
| Camphor . . .           | W   | —   | W   |     |             |                       |    |        |    |             |    |        | M  | B   | S  |
| Pulegone . . .          | MS  | —   | MS  |     |             |                       |    |        |    |             | B  |        | S  | S   | MS |
|                         |     |     |     |     | B           | the most abundant ion | MS | 50-65% | S  | 65-80% of B | W  | 15-30% | VW | 15% |    |
|                         |     |     |     |     | VS          | 80-90% of B           | M  | 30-50% |    |             |    |        |    |     |    |



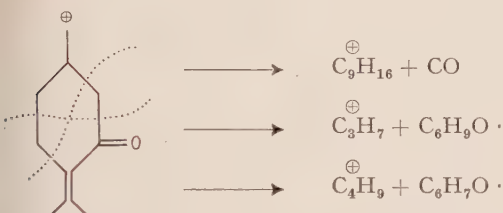
system does not seem to do so in these systems. This is, however, observed in the break-down of the camphane molecular ion



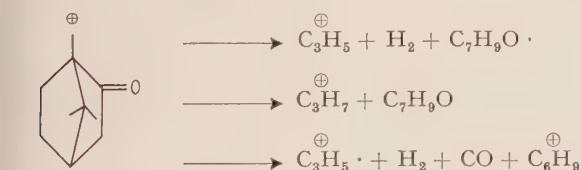
where the ions of masses 95, 43, and 41 are very abundant. Ring-strain seems an important factor in all these systems as the presence of the *gem*-dimethyl bridge system gives a conformational rigidity to the molecule. This is supported by the fact that the elimination of 43 units from 1-methyl-4-isopropylcyclohexane while marked is not as intense as in the systems discussed<sup>2</sup>.

Other prominent fragments which occur in certain of the spectra, namely mass 68 in dipentene, and 75 in  $\Delta^3$  carene may readily be interpreted as allylic-bond fissions. The prominent peak of mass 79 in the  $\alpha$ - and  $\delta$ -fenchenes is not readily explained on this basis without postulating hydrogen migrations also and this system is to be further examined.

These studies have been extended to pulegone and to camphor to allow of comparison with the hydrocarbons. In accordance with previous observations<sup>4,5</sup> the predominant process seems to be the elimination of carbon monoxide. The remaining fragments may be obtained as follows



giving fragment ions of 57, 43, and 28. In a similar way camphor yields fragments of masses 41, 43, and 81.



The other significant fragment ion at low energies, 55, is not so readily interpreted.

It is concluded, therefore, that the principal fragment ions obtained in this series of compounds may, with two exceptions, be obtained directly from the carbocyclic skeletons. They do not at least at the low energies used require re-arrangements of this skeletal structure for their interpretation.

We acknowledge with gratitude the provision of a sample of pulegone by Dr. G. BUCHANAN, The University of Glasgow, of  $\Delta^3$  carene by Dr. L. N. OWEN of the Imperial College of Science and Technology, London, and part of the mass-spectrum of camphor by Mr. W. SNEDDEN of The University of Glasgow.

T. GILCHRIST (in part)  
and R. I. REED

Chemistry Department, The University of Glasgow, July 27, 1959.

## Résumé

Les auteurs ont trouvé quelques corrélations entre la formation des ions les plus abondants et la disposition des liaisons-éthyléniques dans les ions moléculaires de quelques terpènes.

<sup>4</sup> J. H. BEYNON, personal communication.

<sup>5</sup> J. H. BEYNON, R. G. LESTER, and A. E. WILLIAMS, A.S.T.M. E-14 Committee on Mass Spectrometry, May 1959.

## The Effects of Nucleases on Photosynthetic CO<sub>2</sub> Fixation

The occurrence of nucleic acids in chloroplasts has been recognised for quite sometime<sup>1</sup> but very little is known about their function, except possibly an involvement in the propagation of chloroplasts. It has been indicated that the activity of RNA<sup>2</sup> could be linked to the synthesis of specific proteins i.e. enzymes involved in photosynthesis<sup>3</sup>. In view of some recent observations on nucleic acid control of protein and polysaccharide syntheses it was considered of interest to study whether and to what extent the CO<sub>2</sub> fixation reactions in photosynthesis are nucleic acid dependent processes.

Young cultures of *Chlorella pyrenoidosa* and *Nostoc muscorum* were used. The cells were centrifuged in the cold in an MSE centrifuge, suspended in phosphate buffer of pH 7.0 and incubated with deoxyribo- or ribonucleases for 2–2.5 h at 37°C. The cells were then centrifuged again, resuspended in phosphate buffer allowed to metabolise NaH<sup>14</sup>CO<sub>3</sub> (obtained from the Radiochemical Centre, Amersham, England) for a further period of 2 h at 25°C. The cells were extracted in hot 80% ethanol, filtered and the residue washed in 80% ethanol. An aliquot of the filtrate was transferred to stainless steel planchets for counting with an end window  $\beta$ -counter and another chromatographed two dimensionally on Whatman No. 1 filter paper with phenol-water butanol acetic water (4:1:1) as the developing solvent<sup>4</sup>. The radioactive areas on the chromatograms were located by exposure to X-Ray films and counts were taken directly on paper. The DNase (1  $\times$  cryst) used was obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A. and RNase (5  $\times$  cryst.) was supplied by Sigma Chemical Co., St. Louis, Missouri, U.S.A.

Nuclease treatment resulted in slight inhibition (less than 10%) of the incorporation of <sup>14</sup>C into the 80% ethanol-insoluble fraction. Examination of the ethanol soluble fraction however revealed rather severe effects on the synthesis and metabolism of <sup>14</sup>CO<sub>2</sub> fixation products. The effect of nuclease treatment on the total incorporation of <sup>14</sup>C into the alcohol-soluble fraction and the more important products of <sup>14</sup>CO<sub>2</sub> fixation during a 2 h photosynthesis is shown in the Table. It appears that the CO<sub>2</sub> fixation process in *C. pyrenoidosa* is more susceptible to nuclease

<sup>1</sup> E. I. RABINOWITCH, *Photosynthesis and related processes*, Vol. 2, Part 2 (Interscience Publishers, Inc., New York 1956).

<sup>2</sup> The following abbreviations have been used: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; DNase deoxyribonuclease; RNase, ribonuclease.

<sup>3</sup> G. BRAWERMAN and E. CHARGAFF, *Biochim. biophys. Acta* 31, 164 (1959).

<sup>4</sup> B. B. BISWAS and S. P. SEN, *Nature* 181, 1219 (1958).



treatments than that in *N. muscorum*. The inhibitory effect of DNase treatment is more severe than that of RNase; with RNase only a 5% inhibition is noted in *N. muscorum*. It may be due to the difference in penetration of nucleases. The distribution of  $^{14}\text{C}$  into the photosynthetic products in both the organisms is however affected by either of the nucleases in varying degrees. Sucrose synthesis appears to be the most affected. Considerable radioactivity was detected in 'the nucleotide area' of the chromatograms of *N. muscorum* and RNase treatment has been found to result in a marked accumulation of the products involved, probably at the expense of sucrose synthesis.  $^{14}\text{C}$  incorporation into aspartic and glutamic acids is stimulated by both the nucleases in *N. muscorum*, particularly by RNase. In *C. pyrenoidosa* DNase markedly retards aspartic acid synthesis but glutamic acid synthesis is severely affected by RNase. Nucleases have also an inhibitory effect on glycine-serine synthesis in both the organisms. Alanine synthesis in *N. muscorum* is totally abolished by the application of RNase in *C. pyrenoidosa* on the other hand there is a slight stimulation. DNase has some inhibitory effect on alanine synthesis in *C. pyrenoidosa*, but not at all in *N. muscorum*. No incorporation of  $^{14}\text{C}$  into malate or citrate is observed in nucleases treated *C. pyrenoidosa*. In *N. muscorum* malate or citrate had only traces of radioactivity and no conclusion can be made regarding the effect of nucleases.

The effect of nuclease treatment on the products of  $^{14}\text{CO}_2$  fixation in *Chlorella pyrenoidosa* and *Nostoc muscorum*  
(Data expressed as % variation from control for each compound)

| Compound                                      | <i>C. pyrenoidosa</i> |                    | <i>N. muscorum</i> |                    |
|-----------------------------------------------|-----------------------|--------------------|--------------------|--------------------|
|                                               | RNase <sup>a</sup>    | DNase <sup>b</sup> | RNase <sup>c</sup> | DNase <sup>d</sup> |
| Nucleotide area <sup>e</sup> . .              | ... <sup>f</sup>      | ...                | + 227              | - 12               |
| Sugar phosphates . .                          | - 44                  | - 50               | ...                | ...                |
| Sucrose . . . . .                             | - 96                  | - 100              | - 76               | - 63               |
| Aspartic acid . . .                           | - 2                   | - 43               | + 255              | + 8                |
| Glutamic acid . . .                           | - 55                  | - 18               | + 109              | + 11               |
| Glycine-Serine . . .                          | - 50                  | - 69               | - 100              | - 100              |
| Alanine . . . . .                             | + 11                  | - 21               | - 100              | 0                  |
| Citric acid . . . . .                         | - 100                 | - 100              | ...                | ...                |
| Malic acid . . . . .                          | - 100                 | - 100              | ...                | ...                |
| Total $^{14}\text{C}$ incorporation . . . . . | - 49                  | - 76               | - 5                | - 29               |

<sup>a</sup> Packed cell volume 0.8 ml, suspended in 10 ml phosphate buffer of pH 7.0 containing 7 mg RNase. Incubation at 37°C for 2.5 h. After incubation washed, centrifuged, and resuspended in 5 ml phosphate buffer, cells allowed to metabolise 50  $\mu\text{C}$   $\text{NaH}^{14}\text{CO}_3$  for 2 h in light from two 1000 W photoflood lamps 18" away from the incubation mixture, one on either side.

<sup>b</sup> Same as above except that 4.3 mg DNase was added.

<sup>c</sup> 20 ml cell suspension; 5.5 mg RNase, incubation period 2 h after washing and centrifugation resuspended in 5 ml phosphate buffer and incubated with 50  $\mu\text{C}$   $\text{NaH}^{14}\text{CO}_3$  for 2 h otherwise same as above.

<sup>d</sup> 10 ml suspension, 5 mg DNase, resuspended in 6.5 ml phosphate buffer and 100  $\mu\text{C}$  bicarbonate added, otherwise same as above.

<sup>e</sup> The radioactive area near the origin of chromatograms containing nucleotides etc.

<sup>f</sup> Low counts.

From the observations recorded here it appears that both RNase and DNase affect the synthesis and metabolism of some of the  $\text{CO}_2$  fixation products in photosynthesis, particularly sucrose. The response to pretreatment with nucleases for other compounds is varied and the effects are apparently complicated. The metabolic

interrelationship among the products of photosynthesis except the very early ones is not well understood and it is not possible at present to suggest which of the reactions involved are nucleic acid controlled. It should, however, be borne in mind that the effects observed may not be direct effects of nuclease treatments since whole cells were used and the question of permeability is there though it is reported that ribonucleases of molecular weights about 13000 can penetrate living onion root tip cells<sup>5</sup>.

The experiments with isolated chloroplasts and uses of inhibitors in nucleic acid syntheses will further elucidate the point.

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### Zusammenfassung

Es scheint, dass DNase und RNase bei der photosynthetischen Fixierung des  $\text{CO}_2$  verschiedene und komplizierte Wirkung ausüben. Die Saccharose-Synthese wird durch die Behandlung mit DNase und RNase deutlich vermindert.

<sup>5</sup> J. BRACHET, Nature 174, 876 (1954).

<sup>6</sup> B. P. KAUFMAN and N. K. DAS, Proc. nat. Acad. Sci., Wash. 40, 1052 (1954); Chromosoma 7, 19 (1955).

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### Cystine Monosulfoxide

Several pathways for the metabolism of cystine and cysteine are still under consideration<sup>1</sup>. Among the most probable intermediates in the metabolism of cysteine and cystine, only the cystine monosulfoxide has been neither isolated nor synthesized. Its preparation was unsuccessfully attempted by TOENNIES and LAVINE<sup>2-4</sup>. Cystine monosulfoxide is of growing theoretical interest since simple thiosulfinic esters (monosulfoxides of alkyl disulfides) studied by WEISBERGER and PENSKY<sup>5</sup> were shown to possess cancer blocking activity, which these authors derived from the inhibiting effect of allicin on sulfhydryl (-SH) containing enzymes. Allicine (allylthiosulfinic allyl ester) is an enzymic metabolite of alliin, the active principle of garlic (*Allium sativum*)<sup>6-8</sup>.

L-Cystine monosulfoxide has now been prepared by reduction with hydriodic acid in the cold of cystine disulfoxide. The latter had been synthesized in nonaqueous solvent by TOENNIES and LAVINE<sup>3</sup>.

In contrast to the disulfoxide, the monosulfoxide is stable in the presence of 0.5 N NaOH. It darkens and slowly decomposes upon heating above 175°C,  $[\alpha]_D^{25} = -111$  ( $\pm 3^\circ$ ),  $c = 0.70\%$  in N HCl. The ninhydrin spot appears immediately and is deeper colored than the one given by

<sup>1</sup> L. YOUNG and G. A. MAW, The Metabolism of Sulphur Compounds, Chapter V (Methuen Co. Ltd., London 1958), p. 97.

<sup>2</sup> G. TOENNIES, J. Amer. chem. Soc. 56, 2198 (1934).

<sup>3</sup> G. TOENNIES and T. F. LAVINE, J. biol. Chem. 113, 576 (1936).

<sup>4</sup> T. F. LAVINE, J. biol. Chem. 113, 584 (1936).

<sup>5</sup> A. S. WEISBERGER and J. PENSKY, Cancer, Res. 18, 1301 (1958).

<sup>6</sup> C. J. CAVALITO, J. BUCK, and C. SUTER, J. Amer. chem. Soc. 66, 1952 (1944).

<sup>7</sup> A. STOLL and E. SEEBECK, Helv. chim. Acta 31, 189 (1948).

<sup>8</sup> E. D. WILLS, Biochem. J. 63, 514 (1956).

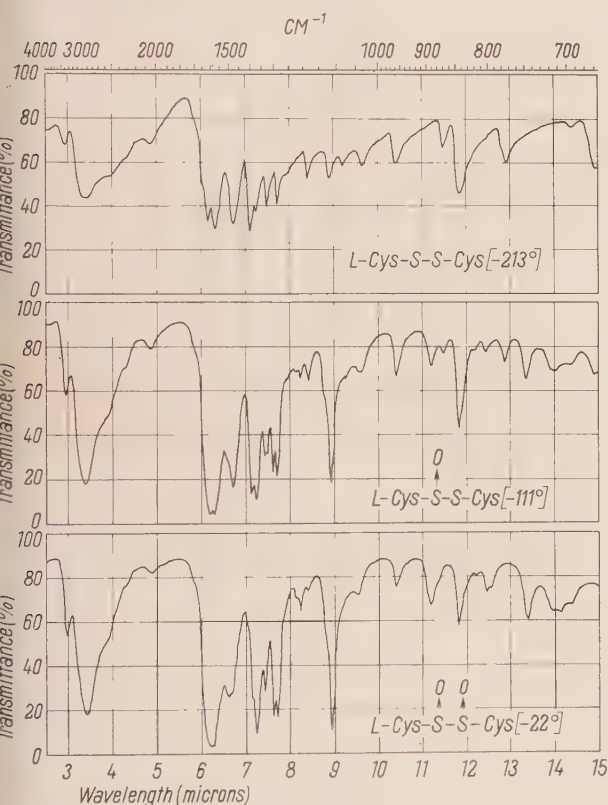


cystine. Contrary to the conclusion drawn by TOENNIES and LAVINE<sup>3</sup> from oxidation mixtures, the monosulfoxide of cystine does respond to the cyanide nitro prusside test<sup>9</sup> although slowly (3–5 min at room temperature). This indicates that the sulfur of the sulfoxide group is still available to nucleophilic attack by the cyanide ion<sup>10</sup>.

The monosulfoxide slowly reduces dichloro-indophenol *in vacuo* at room temperature but has no effect on methylene blue in either its leuko or oxidized form.

The I. R. spectrum (Fig.) of the monosulfoxide is distinguished from the corresponding cystine spectrum by a sharp peak at 8.93  $\mu$  which is assigned to the sulfoxide group<sup>11</sup>. A needle shaped peak at 7.49  $\mu$  allows the discrimination from the disulfoxide, which lacks this peak but shows the usual sulfoxide, peak slightly shifted to 8.95  $\mu$ .

Cystine, as well as its sulfoxides, is relatively immobile with a variety of chromatographic solvents, except in the presence of coal gas and ammonia. Under these conditions, the presence of mercaptan permits the existence and chromatographic movement of monosulfur derivatives of the disulfides and their sulfoxides. These would recombine in the presence of air. We could confirm the ratio of the Rf values reported by WINEGARD *et al.*<sup>12</sup> for cystine (0.25) and its disulfoxide (0.21), but found the mobility in agreement with CONSDEN *et al.*<sup>13</sup> 0.13 and 0.11, respectively. The cystine monosulfoxide could not be discriminated chromatographically from the disulfoxide. Its Rf value in phenol/H<sub>2</sub>O 4:1 in coal gas/NH<sub>3</sub> on Whatman paper No. 3 is 0.11.



I.R. spectra of cystine, cystine monosulfoxide, and cystine disulfoxide\* in KBr 1:400 measured on a Perkin Elmer Spectrophotometer, Modell 21, with Slave Recorder

\* The structure of 'cystine disulfoxide' is not yet established beyond any doubt. The other alternative is the thiosulfonic ester.

**Experimental.** Crude cystine disulfoxide was prepared by the general method of LAVINE<sup>4</sup>. Due to the presence of acetic anhydride in the oxidizing medium, this product contained mono-N-acetyl-cystine disulfoxide. The N-acetyl impurity precipitated first on neutralizing with ammonium hydroxide a solution of the mixed crystals in *N* HCl. The mother liquor contained further cystine monosulfoxide.

The thus purified L-disulfoxide showed  $[\alpha]_D^{25} = -22^\circ (\pm 2^\circ)$ ,  $c = 1.38\%$  in 1 *N* HCl. Lit.<sup>3</sup>  $[\alpha]_D^{25} = -30.2^\circ$  in 1 *N* HCl. L-cystine disulfoxide (2.00 g, 7.5 mmoles) was suspended in 40 ml of water and dissolved with 6 *N* HCl (6 ml 18 mmoles). This solution was cooled in ice and placed in the dark. Five molar KI (3 ml, 15 mmoles) was added and stirred occasionally during  $\frac{1}{2}$  h. The solution was then transferred to a cooled separatory funnel and extracted rapidly 12 times with 30-ml portions of chloroform until the chloroform and the aqueous solution were colorless. The aqueous solution was then neutralized in the cold with 8 *N* NH<sub>4</sub>OH. The precipitate was quickly filtered and washed with alcohol followed by acetone. Yield: 1.38 g (75.2%). Dec. above 175°C. It was stored in an evacuated dessicator.

The automatic titration recorder gave 56 mg =  $4.38 \times 10^{-4}$  mol (2.19 cm<sup>3</sup> 0.2 *N* NaOH): eq. wt. 128 (calculated 128.12).

For analysis the compound was heated 1 h to 100°C in a 1 mm Hg vacuum.

C<sub>6</sub>H<sub>12</sub>N<sub>2</sub>OS<sub>2</sub> 256.24

|              |       |        |         |          |
|--------------|-------|--------|---------|----------|
| Calculated C | 28.10 | H 4.72 | N 10.93 | S 25.02% |
| Found        | 28.34 | 4.93   | 10.66   | 25.17%   |

This work is part of an investigation of the 'iodine trapping syndrome' described by SCOTT *et al.*<sup>14</sup> and supported by Cancer Research funds of the University of California. It was aided by facilities of the School of Pharmacy of the University of California.

I wish to acknowledge my appreciation to Dean T. C. DANIELS and Dr. E. JORGENSEN for their interest in this study.

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### Zusammenfassung

Es wurden die Herstellung und die Eigenschaften von Cystin-monosulfoxid beschrieben und die IR.-Spektren von Cystin, Cystin-monosulfoxid und Cystin-disulfoxid wiedergegeben.

<sup>9</sup> G. TOENNIES and T. F. LAVINE, *J. biol. Chem.* **105**, 115 (1934).

<sup>10</sup> A review on the scission of the disulfide bond was recently published by A. J. PARKER and N. KHARASCH, *Chem. Rev.* **59**, 584 (1959).

<sup>11</sup> H. M. RAUEN'S *Biochemisches Taschenbuch* (Springer-Verlag, Berlin-Göttingen-Heidelberg 1956), lists strong IR peaks from 7.5  $\mu$  to 9.5  $\mu$ .

<sup>12</sup> H. M. WINEGARD, G. TOENNIES, and R. J. BLOCK, *Science* **108**, 506 (1948).

<sup>13</sup> R. CONSDEN, A. H. GORDON, and A. J. P. MARTIN, *Biochem. J.* **40**, 580 (1946).

<sup>14</sup> K. S. SCOTT and C. PENG, *Univ. Calif. Publ. Pharm.* **2**, 345 (1955).



## Action anticaroténogène de la diphénylamine chez l'algue *Chlorella rubescens*

La diphénylamine (DPA) inhibe sélectivement la biosynthèse des caroténoïdes chez la bactérie *Mycobacterium phlei*<sup>1</sup>. Cette action anticaroténogène de la DPA a été confirmée chez les champignons<sup>2,3</sup> et les bactéries photosynthétiques<sup>4,5</sup>. Par contre, chez l'algue *Chlorella vulgaris*, seule une inhibition de croissance a été obtenue<sup>6</sup>.

Nous avons repris la question DPA-algues en utilisant une chlorelle connue pour son pouvoir caroténogène, *Chlorella rubescens* CHOD. et un milieu hétérotrophique propice à la synthèse des caroténoïdes. *Chlorella rubescens* a été cultivée en milieu de DETMER 1/3 enrichi de glucose, avec ou sans (témoins) DPA, réparti dans des ballons phycogènes aérés et éclairés (tubes luminescents), en chambre climatisée à 20°C (installation selon CHODAT et BOCQUET<sup>7</sup>).

Dans de telles conditions et déjà après 3 semaines de culture, une nette inhibition de la composante orange de la pigmentation s'est manifestée chez les algues cultivées en présence de DPA. L'extraction et la chromatographie (MgO-celite) des caroténoïdes ont permis la détection d'un composé à fluorescence gris-vert, ayant les caractéristiques adsorptives et spectrales du phytofluène, dans les cultures traitées à la DPA. Nos premiers résultats sont résumés dans le tableau suivant :

Cultures de *Chlorella rubescens* CHOD. âgées de 4 semaines

| Milieu de DETMER<br>1/3 + source de C | DPA        | Pigmentation:<br>chlorophylles<br>et $\beta$ -carotène-<br>xanthophylles | Composé<br>fluorescent:<br>phytofluène |
|---------------------------------------|------------|--------------------------------------------------------------------------|----------------------------------------|
| Glucose 2% . . .                      | 0          | rouge-orange                                                             | —                                      |
| Glucose 2% . . .                      | 1/200 000  | jaune-vert                                                               | +                                      |
| Glucose 2% . . .                      | 1/125 000* | vert jaunâtre                                                            | +                                      |

\* Cultures de 12 semaines

Dans les observations faites *de visu*, il n'est apparu aucun effet notoire de destruction photodynamique des cellules de *Chlorella* semblable à celui décrit chez les bactéries photosynthétiques<sup>5</sup>. Signalons cependant que KANDLER et SCHÖTZ<sup>8</sup> ont observé une photosensibilité chez leur mutant apo-carotène de *Chlorella*. De nouvelles expériences seront nécessaires pour éclaircir ce point en tenant compte du régime auto- ou hétérotrophique des algues apocaroténoïdes.

Concernant le mode d'action de la DPA, nous avons avancé la possibilité d'une action antioxydante de cette molécule, protégeant les précurseurs polyéniques incolores plus saturés d'une deshydrogénation en polyènes colorés<sup>1,9</sup>. Dans cet ordre d'idées, nous avons observé que la dissolution de quelques cristaux de DPA dans une solution éthanolique de  $\beta$ -carotène protège le pigment d'une rapide photo-oxydation décolorante. Cette expérience démontre le pouvoir antioxydant direct de la DPA à l'égard du  $\beta$ -carotène. De plus, et fait significatif, la DPA sulfonée (hydrosoluble) s'est montrée inactive, en particulier avec les champignons caroténogènes (expériences inédites avec *Allomyces*). Cela tendrait à prouver que l'effet anticaroténogène de la DPA (hydro- et liposoluble) dépend de la faculté particulière de cette molécule de se concentrer aux interfaces phase aqueuse-phase lipidique du cytoplasme et d'y exercer son action antioxydante.

Or, c'est précisément à ce niveau que la deshydrogénation enzymatique des précurseurs polyéniques doit avoir lieu préalablement à l'accumulation des produits finals, les caroténoïdes colorés, dans la phase lipidique des chloroplastes ou dans les gouttelettes lipidiques intracellulaires.

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13 octobre 1959.*

### Summary

Diphenylamine (DPA). inhibition of carotenoid biosynthesis with concomitant appearance of detectable amounts of the colourless and fluorescent polyene, phytofluene, is reported in *Chlorella rubescens* CHOD.

Preliminary negative results with the hydrosoluble DPA-sulphonic acid suggest that anticarotenogenic activity of the hydro- and liposoluble DPA may well be related to the faculty of this molecule to accumulate at the aqueous-lipidic interphases in the cytoplasm where it would interfere with the enzymatic dehydrogenation of the carotenoid precursors through its noted antioxidant properties.

<sup>1</sup> G. TURIAN, *Helv. chim. Acta* 33, 1988 (1950).

<sup>2</sup> G. TURIAN, *Exper.* 8, 302 (1952).

<sup>3</sup> T. W. GOODWIN, *Biochem. J.* 50, 550 (1952).

<sup>4</sup> T. W. GOODWIN et H. G. OSMAN, *Biochem. J.* 56, 222 (1954).

<sup>5</sup> G. COHEN-BAZIRE et R. Y. STANIER, *Nature* 181, 250 (1958).

<sup>6</sup> T. W. GOODWIN, *Exper.* 10, 213 (1954).

<sup>7</sup> F. CHODAT et G. BOCQUET, *Arch. Sci. Genève* 8, 214 (1955).

<sup>8</sup> O. KANDLER et F. SCHÖTZ, *Z. Naturf.* 11b, 708 (1956).

<sup>9</sup> G. TURIAN, *Physiol. Plantarum* 10, 667 (1957).

## On the Conformation of Substituted Cyclopentanones.

### Rotatory Dispersion and Spectral Data of some Steroidal $\alpha$ -Halocyclopentanones<sup>1</sup>

The problem of the conformation of the cyclopentanone ring has been the subject of a number of recent investigations<sup>2-4</sup> and the present status is particularly well summarized by BRUTCHER *et al.*<sup>4</sup>. Of special pertinence is the conformation of the cyclopentanone system, since the corresponding  $\alpha$ -halo derivatives can be examined by a number of physico-chemical tools, which can offer valuable information on the nature of the C-halogen bond and hence on the conformation of the cyclopentanone ring itself. BRUTCHER<sup>4</sup>, on the basis of infrared, nuclear magnetic resonance and dipole moment studies differentiates between three types of bonds: (i) the classical axial and equatorial bonds, found in the cyclohexane system; (ii) quasi-axial and quasi-equatorial bonds, and (iii) a bisecting bond, which is intermediate between axial and

<sup>1</sup> Paper XXXV in the series *Optical Rotatory Dispersion Studies* by C. D. For the preceding article see N. L. ALLINGER, R. B. HERMANN, and C. DJERASSI, *J. org. Chem.*, in press.

<sup>2</sup> C. G. LE FEVRE and R. J. W. LE FEVRE, *J. chem. Soc.*, 354 (1956).

<sup>3</sup> K. S. PITZER and W. E. DONATH, *J. Amer. chem. Soc.* 81, 321 (1959).

<sup>4</sup> F. V. BRUTCHER, T. ROBERTS, S. J. BARR, and N. PEARSON, *J. Amer. chem. Soc.* 81, 4915 (1959) and earlier papers.



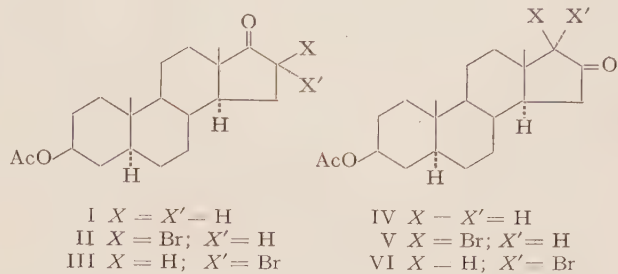
equatorial. The latter type would be found in the  $\alpha$ -positions of a planar cyclopentanone and the  $\alpha$ -halocamphors were employed<sup>4</sup> as suitable reference samples for this purpose, since the ring fusion in this molecule requires virtual planarity of the five-membered ring. The evidence employed so far seems to point<sup>2,4</sup> towards a 'half-chair' conformation of cyclopentanone.

We should now like to present additional evidence bearing on this problem. SHOPPEE *et al.*<sup>5</sup> have already noted that steroidal ring D ketones would represent excellent test cases since the C/D *trans*-ring fusion requires a fixed conformation of the cyclopentanone ring.

The only bromo ketones of this type, which are known<sup>5,6</sup> are the 16 $\beta$ -bromo (II) and 16 $\alpha$ -bromo (III) derivatives of 5 $\alpha$ -androstan-3 $\beta$ -ol-17-one acetate (I) and their infrared and optical rotatory dispersion<sup>7</sup> data (Table) show that the 16 $\alpha$ - and 16 $\beta$ -bromo substituents occupy bisectonal bonds. Considerably more significant are the hitherto undescribed epimeric 17-bromo-16-ketones (V, VI) since in these substances, the two bonds should be non-equivalent. The 17 $\alpha$ -bromo isomer VI has now been prepared<sup>8</sup> in pure form (m. p. 168–172°C) by bromination (carbon tetrachloride solution) of the enol acetate of 5 $\alpha$ -androstan-3 $\beta$ -ol-16-one acetate (IV) followed by chromatographic separation on alumina. The relevant infrared, ultraviolet, and optical rotatory dispersion<sup>9</sup> data are collected in the Table. The predominant product, 17 $\alpha$ -bromo-5 $\alpha$ -androstan-3 $\beta$ -ol-16-one acetate (VI) should – according to the 'half-chair' form<sup>4</sup> – exhibit quasi-axial character. This is borne out by the infrared shift (8 cm<sup>-1</sup>) which is smaller<sup>4</sup> than the bisectonal value (12–14 cm<sup>-1</sup>) and especially by the rotatory dispersion curve. The first extremum of its negative Cotton effect shows a bathochromic shift of 36 m $\mu$  as compared to the trough of the parent ketone (IV), a behavior qualitatively reminiscent<sup>10</sup> of axial  $\alpha$ -bromo cyclohexanones. Furthermore, if the 'axial  $\alpha$ -haloketone'<sup>10</sup> is also applicable<sup>11</sup> to cyclopentanones, then a 17 $\alpha$ -bromo substituent would be expected to make a positive rotatory contribution and this is clearly the case here. Similar conclusions can also be derived from the reported<sup>12</sup> spectral and rotatory dispersion shifts of 17-acetoxy-16-keto steroids.

The results cited so far are consistent with the half-chair conformation of the cyclopentanone ring. It is pertinent to mention that our results summarized in the Table indicate that ultraviolet spectral measurements<sup>13</sup> may not always represent a conclusive criterion in the cyclopentanone series. Thus the ultraviolet bathochromic shift (16.5 to 23 m $\mu$ ) for an  $\alpha$ -bromocyclopentanone of the bisectonal type is unexpectedly larger than that exhibited (17 m $\mu$ )

by a quasi-axial  $\alpha$ -bromo ketone (VI), while the anticipated<sup>10</sup> reverse relationship is shown by the rotatory dispersion data.



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Zusammenfassung

Die optische Rotationsdispersion sowie das Infrarot- und Ultraviolett-Absorptionsspektrum von mehreren  $\alpha$ -Bromderivaten der 16-Keto- und 17-Ketosteroide wurde gemessen. Die Schlussfolgerungen aus Rotationsdispersion und Infrarotanalyse stimmen gut überein, es scheint jedoch, dass die Verschiebungen im Ultraviolett zur Bestimmung der Orientierung eines Halogenatoms in  $\alpha$ -Bromocyclopentanonen nicht in allen Fällen gebraucht werden können.

<sup>5</sup> C. W. SHOPPEE, R. H. JENKINS, and G. H. R. SUMMERS, J. chem. Soc., 3048 (1958).  
<sup>6</sup> J. FAJKOS, Chem. Listy 48, 1800 (1954); 49, 1218 (1955); J. Chem. Soc., 3966 (1959).  
<sup>7</sup> C. DJERASSI, *Optical Rotatory Dispersion: Applications to Organic Chemistry* (McGraw Hill Book Co., New York 1960).  
<sup>8</sup> J. FISHMAN, to be published.  
<sup>9</sup> The rotatory dispersion results are reported in terms of the first extremum of the Cotton effect and as pointed out in Chap. 8 of ref. <sup>7</sup>, the wavelength differences between the parent ketone and its  $\alpha$ -substituted derivative permit direct comparison with the corresponding ultraviolet absorption measurements (see also ref. <sup>12</sup>).  
<sup>10</sup> C. DJERASSI and W. KLYNE, J. Amer. chem. Soc. 79, 1506 (1957). – C. DJERASSI, J. OSIECKI, R. RINIKER, and B. RINIKER, J. Amer. chem. Soc. 80, 1216 (1958).  
<sup>11</sup> That this appears to be the case is indicated by A. LARDON, H. P. SIGG, and T. REICHSTEIN, Helv. chim. Acta 42, 1462–1463 and Fig. 1 (1959).  
<sup>12</sup> C. DJERASSI, O. HALPERN, V. HALPERN, O. SCHINDLER, and C. TAMM, Helv. chim. Acta 41, 250, especially Table 4 (1958).

| Substance                                    | I.R. $\nu_{\text{max}}^{\text{CCl}_4}$<br>(cm <sup>-1</sup> ) | $\Delta\nu$<br>(cm <sup>-1</sup> ) | U.V. $\lambda_{\text{max}}^{\text{EtOH}}$<br>(m $\mu$ ) | $\Delta\lambda$<br>(m $\mu$ ) | R.D. first extremum<br>$\lambda_{\text{MeOH}}$<br>(m $\mu$ ) | R.D. $\Delta\lambda$<br>[ $\alpha$ ]<br>(m $\mu$ ) |
|----------------------------------------------|---------------------------------------------------------------|------------------------------------|---------------------------------------------------------|-------------------------------|--------------------------------------------------------------|----------------------------------------------------|
| Camphor . . . . .                            | 1744                                                          |                                    | 289.5                                                   |                               | 310                                                          | + 2000°                                            |
| 3 $\alpha$ -Bromocamphor . . . . .           | 1758                                                          | 14                                 | 306                                                     | 16.5                          | 335                                                          | + 1970°                                            |
| 17-Ketone (I) . . . . .                      | 1743                                                          |                                    | 293                                                     |                               | 315                                                          | + 2700°                                            |
| 16 $\beta$ -Bromo-17-ketone (II) . . . . .   | 1755                                                          | 12                                 | 316 <sup>a</sup>                                        | 23                            | 341.5                                                        | + 2097°                                            |
| 16 $\alpha$ -Bromo-17-ketone (III) . . . . . | 1755                                                          | 12                                 | 316 <sup>a</sup>                                        | 23                            | 341.5                                                        | + 1290°                                            |
| 16-Ketone (IV) . . . . .                     | 1746                                                          |                                    | 299                                                     |                               | 314                                                          | – 4782° <sup>b</sup>                               |
| 17 $\alpha$ -Bromo-16-ketone (VI) . . . . .  | 1754                                                          | 8                                  | 316                                                     | 17                            | 350                                                          | – 525°                                             |

<sup>a</sup> When measured in methanol solution, the maximum occurred at 314–315 m $\mu$ . <sup>b</sup>The free alcohol was used for this determination. All measurements were conducted in our laboratories except for the infrared (ref. <sup>4</sup>) and ultraviolet (ref. <sup>13</sup>) data of camphor and 3 $\alpha$ -bromocamphor.







The last group G was given, immediately after infection, 5 mg indole subcutaneously every other day for 30 days. The experimental period was two months, after which the animals were sacrificed. During indole administration it was found that a daily 25 mg dose was fatal to the guinea pigs. For this reason the 5 mg dose was chosen to be given every other day to secure no harmful effect. All animals were examined at autopsy and, in all, touch smears from sections of lymph nodes, spleen, liver, and lungs were made, stained, and examined.

The *postmortem* examination of the control animals showed the spread of infection, manifested by enlargement and caseation of the regional lymph glands at the site of inoculation with generalization, especially in the liver and spleen which were markedly enlarged and full of macroscopic miliary nodules all over. Films made and stained with Ziehl-Neelsen and examined microscopically revealed the presence of the tubercle organism. On the other hand, the animals treated with INH and streptomycin showed no macroscopic lesions; the spleens, livers, and lymph nodes were normal. Microscopic examination proved negative for T. B. The animals treated with indole were similar to those treated with INH and streptomycin showing no lesions. The regional lymph nodes were markedly fibrosed, and microscopic examination of the films showed negative results for T. B.

Indoleacetic acid and indolepropionic acid showed also a certain activity in limiting the spread of tuberculous infection. The lesions in the spleen and liver were much less than in the control animals. The regional lymph nodes, on the other hand, were similar to those of the control animals. Indoleacetic acid was slightly more effective than the indolepropionic acid.

The most interesting finding here is the tuberculostatic effect of indole in therapeutic dose (5 mg per animal) comparable with that of the INH. Indole itself shows this tuberculostatic effect *in vitro* and interferes also with the utilisation of asparagine for the growth of the tubercle organism. The tuberculostatic effect of indoleacetic acid and indolepropionic acid *in vitro* may be due to the oxidation of the side chain by the microorganism which leads to indole production, and this in turn causes the apparent inhibition. The same mechanism of producing indole might take place in the tissue cells of the guinea pig. This might explain the slight tuberculostatic effect of these compounds. It seems possible also that the lengthening of the side chain in the 3-position of the indole ring lessens the tuberculostatic effect of the indole derivative. This is shown from the higher activity of indoleacetic acid than the indolepropionic acid. Of course this suggestion requires confirmation by examining different indole derivatives with longer side chain in the 3-position of the indole ring. The main drawback of indole is its toxicity. ETS and FEINBERG<sup>7</sup> obtained paralysis of the dog jejunum by injecting intravenously 25 or 60 mg indole per kg body weight. It is considered that indole has a depressant effect due to a direct action on the smooth muscle fibres<sup>8</sup>. In fact, indole in 100 mg/kg body weight was fatal to the guinea pig.

**Acknowledgment.** We wish to thank the Scientific Department of Hoffmann-La Roche for their generous supply of indole derivatives, and Prof. Dr. A. SAEED of the Organic Chemistry Department, Faculty of Science, Cairo University, for provision of indole.

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## Résumé

L'étude des effets de l'indole sur l'organisme *M. tuberculosis hominis* révéla qu'il est bactériostatique *in vitro* et *in vivo* chez les cobayes infectés par lui (5 mg par jour pendant 15 jours). Les acides indol-acétiques et indol-propioniques sont aussi légèrement tuberculostatiques.

<sup>7</sup> H. N. ETS and I. M. FEINBERG, Amer. J. Physiol. 136, 646 (1942).

<sup>8</sup> J. A. IZQUIERDO and A. O. M. STOPPANI, Brit. J. Pharmacol. 8, 389 (1953).

## Cholesterin und Fettsäuren als Ersatz von Serum in der Kultur von *Trichomonas foetus*

Für Trichomonaden wurden halbsynthetische Medien entwickelt, die Serum, Trypticase und Agar als nicht definierte Bestandteile benötigen<sup>1,2</sup>. Neben diesen enthalten sie eine Vielzahl von organischen und anorganischen Stoffen, deren Bedeutung im einzelnen nicht völlig abgeklärt ist. Unter diesen Bedingungen scheint dem Serum der Charakter eines essentiellen Wachsfaktors zuzukommen.

Nach Untersuchungen von SPRINCE<sup>3</sup> sind zwei Fraktionen von Rinderserum wichtig, eine wasserlösliche und eine ätherlösliche. Von den in der ätherlöslichen Fraktion vorhandenen Stoffen gilt Cholesterin als wesentlich<sup>2,4,5</sup>. Die quantitativen Verhältnisse in Beziehung zur wasserlöslichen Fraktion und die Bedeutung anderer lipoidlöslicher Stoffe sind vorläufig nicht genügend abgeklärt<sup>6</sup>. Aus diesem Grunde wurde versucht, zunächst die ätherlösliche Fraktion aus Serum weiter zu charakterisieren. Es wurden vor allem Cholesterin und Cholesterin-Fettsäure-Mischungen geprüft.

Das Ergebnis der Versuche mit *T. foetus* ist folgendes: Auf einem der genannten leicht modifizierten halbsynthetischen Nährböden vermögen Trichomonaden nur in Gegenwart von mehr als 0,3% der wasserlöslichen Fraktion des Serums allein zu wachsen. Bei niedrigeren Konzentrationen ist jedoch mit Zusatz von Cholesterin (10<sup>-5</sup>) ein gleich starkes Trichomonadenwachstum wie mit etwa 1% unfraktioniertem Serum möglich. Die verdünnte wasserlösliche Serumfraktion wird somit durch Cholesterin komplettiert. Cholesterin allein in entsprechenden Konzentrationen besitzt keinen Effekt. Die Frage, welcher Art der wasserlösliche Faktor ist, der Cholesterin als Supplement bedarf, um Trichomonadenwachstum zu gestatten, wurde hier nicht näher verfolgt.

In Gegenwart einer für die Vermehrung der Trichomonaden allein ungenügenden Menge der wasserlöslichen Fraktion (0,3%) ergibt der Zusatz von Fettsäuren teilweise eine beschränkte Vermehrung (vgl. Tabelle), die im Maximum 1/5 der in Gegenwart von Cholesterin (10<sup>-5</sup>) auftretenden Zellzahl erreicht. Hierbei fördern Stearin-, Elaidin- und Ölsäure das Wachstum in Konzentrationen von 10<sup>-5</sup> und weniger, wogegen höher ungesättigte Fettsäuren mit derselben Anzahl C-Atomen (18), nämlich Linol- und Linolensäure, in keiner der geprüften Konzentrationen fördernd wirken. Höhere Konzentrationen der

<sup>1</sup> H. SPRINCE und A. B. KUPFERBERG, J. Bact. 53, 435 (1947).

<sup>2</sup> M. SANDERS, J. Protozool. 4, 118 (1957).

<sup>3</sup> H. SPRINCE und A. B. KUPFERBERG, J. Bact. 53, 441 (1947).

<sup>4</sup> R. CAILLEAU, C. R. Soc. Biol., Paris 127, 861 (1938).

<sup>5</sup> R. GUTHRIE und E. SNELL, Bact. Proc. 28, 44 (1950).

<sup>6</sup> W. WYSS, Diss. Bern (1959).



Fettsäuren hemmen die Vermehrung in unterschiedlichem Masse. Die geprüften Fettsäuren vermögen somit Cholesterin nicht voll zu ersetzen.

Es scheint deshalb notwendig zu untersuchen, ob sie in Gegenwart von Cholesterin ( $10^{-5,0}$ ) eine andere Wirkung besitzen. Das Ergebnis dieser Versuche ist folgendes (Tabelle): Mit Ausnahme von Stearinsäure geben alle geprüften Fettsäuren in einem gewissen niedrigen Konzentrationsbereich (maximal bei  $10^{-5,5}$ ) eine 1,7- bis 3,5mal höhere Zellzahl als Cholesterin allein. Hier haben also auch Linol- und Linolensäure, die ohne Cholesterin sonst nur hemmend wirken, einen die Vermehrung begünstigenden Effekt. Hohe Konzentrationen der Fettsäuren, Elaidin- und Stearinsäure ausgenommen, wirken auch in Gegenwart von Cholesterin im allgemeinen hemmend.

Die Wirkung von Fettsäuren auf die Vermehrung von *T. foetus*

| Fettsäure                                                   | Wuchswirkung (Vielfaches der Zellzahl der Kultur ohne Fettsäure) |           |                               |           |
|-------------------------------------------------------------|------------------------------------------------------------------|-----------|-------------------------------|-----------|
|                                                             | ohne Cholesterol                                                 |           | mit Cholesterol ( $10^{-5}$ ) |           |
|                                                             | Max.                                                             | Min.      | Max.                          | Min.      |
| <i>n</i> -Octadecan-säure (Stearin-)                        | 2 × (5,0)                                                        |           |                               |           |
| <i>trans-n</i> -Octadec-9-en-säure (Elaidin-)               | 3,2 × (6,5)                                                      |           | 3,5 × (5,5)                   |           |
| <i>cis-n</i> -Octadec-9-en-säure (Öl-)                      | 3,9 × (5,0)                                                      |           | 2,8 × (5,5)                   | 0,6 (4,5) |
| <i>cis-cis-n</i> -Octadec-9,12-dien-säure (Linol-)          |                                                                  | 0,6 (4,5) | 2,5 × (5,5)                   | 0,1 (4,5) |
| <i>cis-cis-cis-n</i> -Octadec-9,12,15-dien-säure (Linolen-) |                                                                  | 0,3 (4,5) | 1,7 × (5,5)                   | 0,3 (4,5) |

In Klammern steht die Konzentration der Fettsäure als -log

Die Frage nach den Komponenten, die in der ätherlöslichen Serumfraktion in Gegenwart von wasserlöslicher Fraktion die Vermehrung der Trichomonaden fördern, ist somit in folgender Weise zu beantworten: Cholesterin zeigt eine Wuchsstoffwirkung, gewisse Fettsäuren allein sind ebenfalls, aber viel weniger wirksam als Cholesterin. In Gegenwart von Cholesterin vermögen ungesättigte Fettsäuren, sogar höher ungesättigte wie Linol- und Linolensäure, die Vermehrung anzuregen. Mit Cholesterin plus einzelne Fettsäuren wird eine Wuchswirkung erzielt, die mindestens gleich gut ist wie die mit der ätherlöslichen Lipidfraktion.

Ohne dass auf Grund dieser Versuche entschieden werden kann, ob im Cholesterin und den Fettsäuren bereits sämtliche für die Wuchswirkung massgebenden Bestandteile der ätherlöslichen Fraktion erfasst sind, zeigen sie, dass wahrscheinlich mehrere im Serum normalerweise vorkommende lipoide Stoffe für den Effekt dieser Fraktion verantwortlich sind und dass der Grad der Wuchswirkung in starkem Masse von den quantitativen Mischungsverhältnissen solcher Komponenten abhängig ist.

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Forschungslaboratorien der CIBA Aktiengesellschaft, Pharmazeutische Abteilung, Basel, 14. Januar 1960.

### Summary

In the presence of a minimal amount of a water-soluble growth factor from bovine serum, cholesterol together with an unsaturated fatty acid may replace the ether-soluble lipid fraction of the same serum. The concentration of cholesterol and fatty acid, as well as the chemical structure of the latter, are relevant.

## Elektronenoptische Untersuchungen über die Entstehung «stachliger» Streptomyces-Sporen

Die unterschiedliche Sporenform verschiedener Streptomyces-Arten, die FLAIG *et al.*<sup>1</sup> elektronenoptisch erstmalig nachweisen konnten, ist mehrfach bestätigt worden. Auch über die Bildung typischer Streptomyces-Sporen, die sich innerhalb der Luftmycelmembran – also *endogen* – entwickeln, herrscht prinzipiell Übereinstimmung. Eine verschiedenartige Interpretation hingegen findet der Prozess der Freisetzung dieser Sporen. So neigt ENGHUSEN<sup>2</sup> zu der Meinung, dass «alle Streptomyces-Sporen nach genügender Reife eine Sporenhülle verlassen», ohne auch nur den grundlegenden Mechanismus anzudeuten. Vorstellungen von VERNON<sup>3</sup> zufolge hat man sich die Sporenfreisetzung aus nicht zum Verfall neigenden Hüllen «by means of a longitudinal split» vorzustellen. Letzteres scheint jedoch nicht für alle Arten der Gattung Streptomyces zuzutreffen, vielmehr liegt häufig dem Entstehen von Einzelsporen ein einfacher Modus zugrunde, der im Zerfall der eingeschnürten Luftmycelhyphen beruht, so wie ihn schon LACHNER-SANDOVAL<sup>4</sup> andeutete und wie er von FLAIG *et al.*<sup>5</sup> bestätigt wurde.

Die vorliegenden Untersuchungen wurden an einem Streptomyces-Stamm der morphologischen Sektion «Spiral»<sup>6</sup> (PRIDHAM, HESSELTINE und BENEDICT<sup>7</sup>) ausgeführt, der charakteristisch stachelige Sporen besitzt und sollen einen Beitrag zur Entstehung und Natur dieser Sporenstacheln liefern. Schon nach 48stündiger Kultur des Stammes auf Stärkeagar bei 28°C liessen sich im Kontaktpräparat elektronenoptisch neben glatten, mässig bestachelte, sonst aber noch undifferenzierte Hyphen erkennen (Abb. 1a). Nach weiteren 24–48 h zeigten die Hyphen ausgeprägtere Stachelbildung, und gleichzeitig konnten solche mit beginnender Einschnürung beobachtet werden (Abb. 1b und c). Im Laufe des Wachstums kam es zum Zerfall dieser Hyphen und zur Bildung von Einzelsporen, die auch in unmittelbarer Nähe der ehemaligen Verbindungsstellen Stacheln trugen (Abb. 2a). Die Stacheln scheinen hierbei keine Auswüchse der Sporenmembran zu sein, sondern sind offenbar aus der Membran der Luftmycelhyphen hervorgegangen. Da die Zellwände junger Hyphen des Streptomyces-Lysozym-anfällig sind, lag es nahe, den Abbau der Stacheln zu versuchen. Behandelte man die Sporen mit Lysozym

<sup>1</sup> W. FLAIG, H. BEUTELSPACHER, E. KÜSTER und G. SEGLER-HOLZWEISSIG, *Plant and Soil* 4, 118 (1953).

<sup>2</sup> H. ENGHUSEN, *Arch. Mikrobiol.* 21, 329 (1955).

<sup>3</sup> T. R. VERNON, *Nature* 176, 935 (1955).

<sup>4</sup> V. LACHNER-SANDOVAL, *Diss. Strassburg*, 1898.

<sup>5</sup> W. FLAIG, E. KÜSTER und H. BEUTELSPACHER unter Mitwirkung von I. SCHLICHTING-BAUER, W. POLITT-RUNGE und R. KURZ, *Zbl. Bakteriol.* II, 103, 376 (1954/55).

<sup>6</sup> Diesen Stamm verdanke ich Herrn Prof. E. BALDACCI, Milano (Italien).

<sup>7</sup> T. G. PRIDHAM, C. W. HESSELTINE und R. G. BENEDICT, *Appl. Microbiol.* 6, 52 (1958).



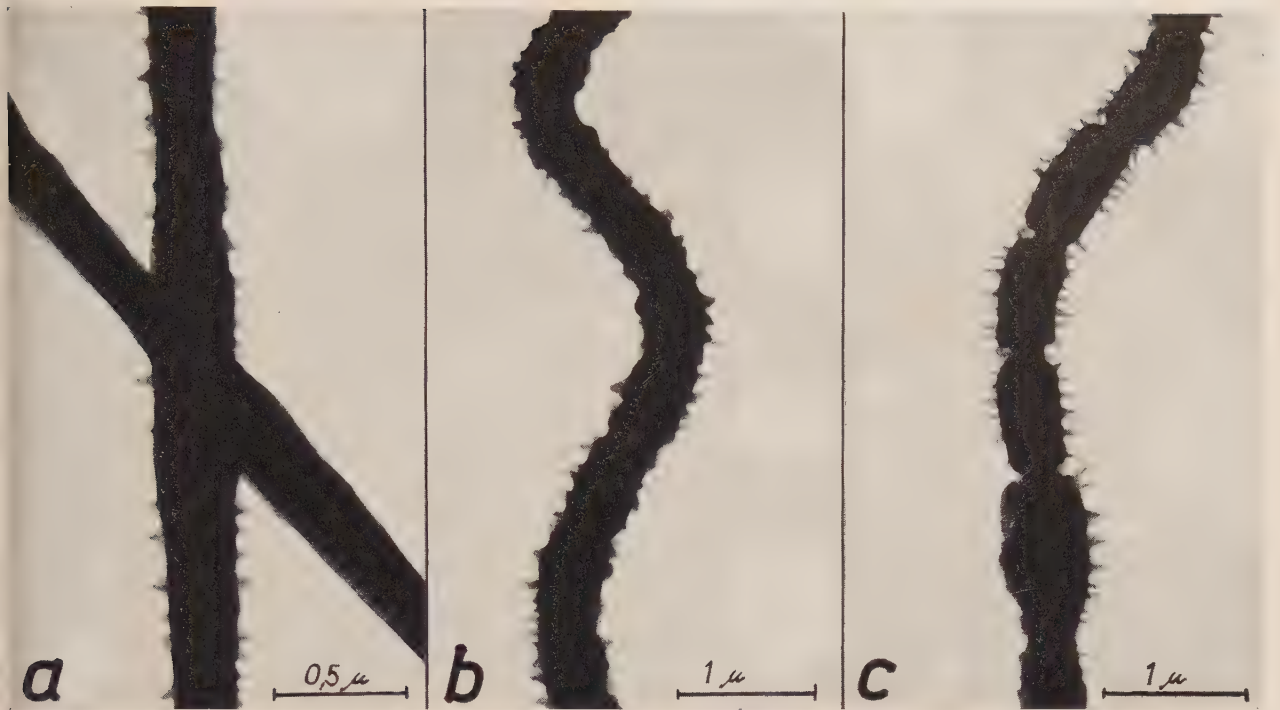


Abb. 1

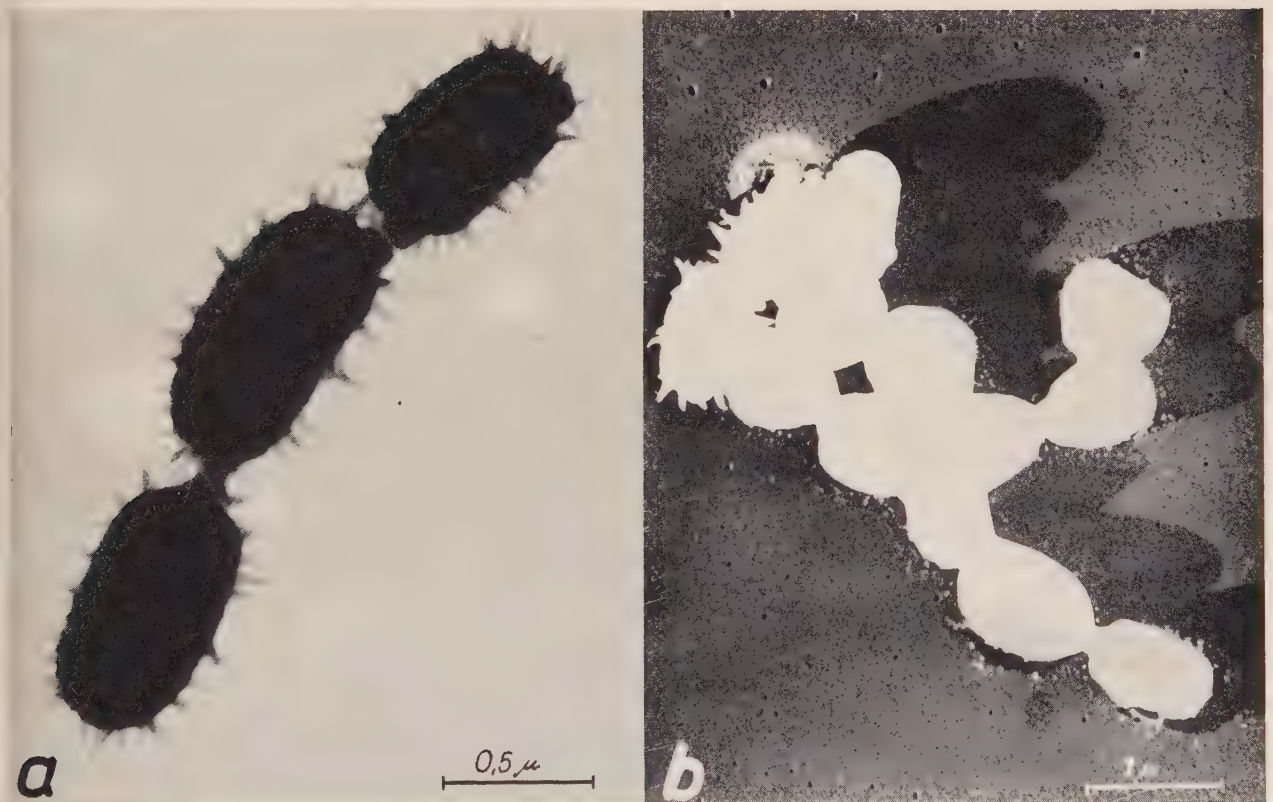


Abb. 2



(Armour & Co.) (100  $\gamma$ /ml in 0,03 M Phosphatpuffer von pH 7,0), so gelang es, die Stacheln abzubauen, ohne dass die Lysozym-resistentere Sporenmembran angegriffen wurde; die Sporen erschienen nach der enzymatischen Behandlung unbestachelt, was als Beweis für die Entstehung der Stacheln aus der Hyphenmembran gewertet wurde (Abb. 2b).

Wie weitere, noch nicht abgeschlossene Untersuchungen zeigten, existiert neben diesem Modus der Stachelbildung ein weiterer, bei dem die Stacheln Auswüchse der Sporenmembran sind. Nach Freiwerden der Sporen bleiben leere, bestachelte Hüllen zurück. Hierbei könnte es sich um Sporen mit mehreren, mindestens aber mit zwei Membranschichten handeln. Eine sichere Entscheidung über die Membranverhältnisse werden Ultradünnschnitte durch verschiedene Sporentypen ergeben.

Dank gebührt meinem Institutsdirektor, Herrn Prof. Dr. BORRIS, für die anregenden Diskussionen, Herrn E. FISCHER für seine Unterstützung bei der Anfertigung der elektronenoptischen Aufnahmen, sowie Fräulein BOLDUAN für ihre gewissenhafte Mithilfe.

F. MACH

Botanisches Institut der Ernst-Moritz-Arndt-Universität zu Greifswald, 7. Mai 1959.

### Summary

It is demonstrated, by electron-optical photography in a *Streptomyces* strain of the morphological section 'spira', that the spines of the spores arise from the membrane of the aerial mycelium hyphae and can be decomposed by lysozyme.

### Localisation of Alkaline Phosphatase in the Development of the Vertebral Column in Chick

The association of the alkaline phosphatase in the mechanism of bone formation has been shown to be an important feature (MOOG<sup>1</sup> and LORCH<sup>2</sup>). A follow-up of the alkaline phosphatase reaction in the development of the vertebral column of chick embryos will be worthwhile because no detailed histochemical study of phosphatase in the chick vertebral column has so far been made, although MOOG<sup>1</sup> gives a general account of phosphatase in embryogenesis of the chick. It is important to bear in mind the changes of phosphatase reaction in the sclerotogenous tissues undergoing histogenesis and forming the elements of the vertebral column.

This study is based on White Leghorn eggs. A series of embryos ranging from 24 h to 17 days of incubation were collected. The total number of embryos studied was 32. They were fixed in chilled 80% alcohol. Serial sections were made of 10  $\mu$  thickness. For the alkaline phosphatase reaction of the cell, a modified technique of Gomori was followed, comparing each reaction with controlled slides.

*Positive homogeneous reaction in the sclerotogenous cells.* In the beginning of the development of the vertebral column, the sclerotogenous cells show a positive homogeneous reaction of phosphatase activity. In the embryos of 24 h and 48 h, the transverse sections show that the whole field is uniformly positive in phosphatase localisation. The cells concentrate round the notochord to form the perichordal tube which is positive in the enzyme reaction. Further development of the perichordal tube is possible by subsequent accumulation of the sclerotogenous cells round the notochord. At this stage, the cells

of the centrum are highly positive in reaction; they seem to be more reactive to this enzyme than those of the neural arches of the two sides. The cells of the basidorsal at the top may be slightly less in enzyme reaction than at its base. The area of the future supradorsal gives a feeble reaction.

*Diminution of the phosphatase reaction.* The changes of the alkaline phosphatase reaction in the cells of the developing centrum and arches become detectable next in the embryos of sixth and seventh day of incubation when the sclerotogenous cells are transformed into protochondrium. The development of the centrum and arches has now become more well defined by the cell aggregations. The most noticeable change in the conversion of the osteogenous cells is the evidence of a decrease of the phosphatase activity in them. At the periphery of the developing vertebra, the surrounding cells continue to show rich phosphatase accumulation. Decrease of phosphatase reaction in the future vertebral elements is well marked. The positive reactions of the peripheral cells are also clear.

Furthermore, the centrum proper, the fibrous arch elements of the neural arch, the fibrous crescent shaped intercentrum and the packing cells continue to be rich in the phosphatase activity.

Whenever there is a secondary plastering of cells, these cells are invariably positive in reaction. The notochordal cells, at this stage, do not show any phosphatase activity. In an embryo of 10 days, sections demonstrate the weak nature of the phosphatase activity.

*Revival of phosphatase activity during calcification.*— In the general background of a reduced phosphatase reaction of the cellular matrix of the centrum and the arches, a number of reactive cells appear. The peripheral cells continue to be positive in the enzyme activity. This marks a transformation of the sclerotogenous cells and a revival of the phosphatase activity in the different elements of the vertebral column. The appearance of phosphatase reaction in the cells coincides with the process of calcification and marrow formation. The reappearance of phosphatase has been observed to be largely extracellular in nature. Gradually the number of reactive areas increases and at the 7<sup>th</sup> day of incubation large number of cells accumulate alkaline phosphatase round them. It shows that the phosphatase activity is noticed around the cells at a time when they are undergoing a process of calcification. Phosphatase-laden osteoid gradually achieves thickness. A number of reactive cells appear among the negative ones in the centre of the centrum and arches of the vertebral column.

*Discussion.*—The important feature of this study centres round the phase-variation of the alkaline phosphatase activity of the cells in the developmental process of the vertebral column. To start with, the sclerotogenous cells show positive alkaline phosphatase reaction. However, this positive reaction gradually fades away to an almost negative degree when the cells reach a stage of protochondrium. The process of losing phosphatase reaction of cells at the protochondrium condition has also been observed previously by MOOG<sup>1</sup> in chick embryos and by LORCH<sup>2</sup> in fish embryos. Reappearance of phosphatase in the cells at a later stage of calcification and marrow formation is a feature of utmost importance. DANIELI<sup>3</sup> has also emphasised this point and has recorded: 'This

<sup>1</sup> F. MOOG, Biol. Bull. 86, 51 (1944).

<sup>2</sup> I. J. LORCH, Quart. J. micr. Sci. 90, 381 (1949).

<sup>3</sup> J. F. DANIELI, Cytochemistry — A Critical Approach (1953), p. 65.



intracellular phosphatase is not associated with bone formation and may largely disappear, to be succeeded by a second wave of formation of phosphatase, a great part of which is found outside the cells'. A point of discrimination is to be made about the nature of phosphatase reactions found in the cells before the disappearance and after the reappearance. Before the disappearance, the alkaline phosphatase localisation is seen to be mostly intracellular, whereas after the reappearance it is extracellular in nature. However, the deep phosphatase localisation of the mesenchymatous cells is throughout an important peculiarity of this study. The plastering cells are mesenchymatous cells and that is why they always show positive phosphatase reaction. The study confirms the view that alkaline phosphatase is associated in the development of the bone element of the vertebral column, and at the time of calcification, there is a phenomenon of reappearance of phosphatase in the cells. This study also supports the view previously expressed by LORCH<sup>2</sup> that, in the absence of extracellular phosphatase, there cannot be formation of bone.

This problem was suggested by Dr. SIVATOSH MOOKERJEE and I am indebted to him for his constant help and encouragement.

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June 19, 1959.

Zusammenfassung

Zu Beginn der Entwicklung der Wirbelsäure im Hühnerembryo findet sich die alkalische Phosphatase intrazellulär. Mit der späteren Verkalkung und Entwicklung des Knochenmarks ist die Reaktion extrazellulär nachweisbar.

Protective Action of Irradiated Bone Marrow Cells

The potential of the host's immunogenic tissue surviving following lethal irradiation determines whether a foreign graft will take. If it is feasible to completely suppress the immunogenic system of the host by x-ray, the immunologic potential of the graft determines how long the host will survive. Provided that the remaining host's immunogenic tissue after lethal irradiation is negligible, 'takes' and progressive growth in the new hosts of foreign bone marrow, if still functional, would ensue<sup>1</sup>. Even if the implants were hypo-functional, or afunctional, they would nevertheless modify the irradiation response.

Since it appears undesirable to inject cells fully capable of reacting immunologically against the host, in the present study, bone marrow was subjected to irradiation *in vitro* prior to transplantation. The degree of protection afforded by isologous (DBA<sub>1</sub>), homologous (C57BL/6) and heterologous (WR) bone marrow exposed to fractionated doses was assessed from the number of mice (DBA<sub>1</sub>) surviving lethally irradiated controls. The effects of all treatments are presented in tabular form (Table).

The observations made indicated that: (1) isologous bone marrow cells exposed *in vitro* up to 950 r exercised some protective effect in lethally irradiated mice, and (2) irradiation of homologous and heterologous bone marrow before injection, (a) did not render them incapable of reaction against the host; (b) were destroyed, or, (c) could not repair the host's radiation-injured cells.

Assuming that radiation is cumulative, the total amount of x-radiation to the animals' bone marrow is obtained simply by adding the doses of lethal body irradiation and the *in vitro* exposure of the isologous bone marrow injected<sup>2</sup>.

<sup>1</sup> G. W. SANTOS and L. J. COLE, J. nat. Cancer Inst. 21, 279 (1958).  
<sup>2</sup> W. SHELDON, K. C. ATWOOD, M. L. RANDOLPH, and H. E. LUIPPOLD, Biophys. biochem. Cytol. 4, 265 (1958).

| Experiment                                      | No. of Mice | Treatment of host; X-irradiation* | Treatment of graft; X-irradiation | M ST of mice dying of exposure | No Survival over Total number |
|-------------------------------------------------|-------------|-----------------------------------|-----------------------------------|--------------------------------|-------------------------------|
| I<br>Isologous bone marrow** . . . . . (DBA/1)  | 5           | 509 r                             | No bone marrow                    | 11                             | 0/5                           |
|                                                 | 5           | 509 r                             | None                              | Permanent                      | 5/5                           |
|                                                 | 5           | 950 r                             | 475 r                             | 28                             | 1/5                           |
|                                                 | 5           | 950 r                             | 950 r                             | 14                             | 1/5                           |
|                                                 | 5           | 950 r                             | 1425 r                            | 10                             | 0/5                           |
| II<br>Homologous bone marrow . . . . . (C57B/6) | 5           | 950 r                             | No bone marrow                    | 11                             | 0/5                           |
|                                                 | 5           | 950 r                             | None                              | 22                             | 2/5                           |
|                                                 | 5           | 950 r                             | 475 r                             | 15                             | 0/5                           |
|                                                 | 5           | 950 r                             | 950 r                             | 9                              | 0/5                           |
|                                                 | 5           | 950 r                             | 1425 r                            | 19                             | 0/5                           |
| III<br>Heterologous bone marrow . . . . .       | 5           | 950 r                             | No bone marrow                    | 11                             | 0/5                           |
|                                                 | 5           | 950 r                             | None                              | 12                             | 0/5                           |
|                                                 | 5           | 950 r                             | 475 r                             | 9                              | 0/5                           |
|                                                 | 5           | 950 r                             | 950 r                             | 10                             | 0/5                           |
|                                                 | 5           | 950 r                             | 1425 r                            | 10                             | 0/5                           |

\* LD<sub>100/11</sub> = 950 r; rate 190 r/min. 250 Kvp, 15 ma; 1/2 mm Cu + 1 mm Al filters    \*\* 10<sup>8</sup>-10<sup>12</sup> cells/0.5 ml saline i.v. 24 h post-irradiation



The fact that *isologous* marrow exposed to 1425 r still contained viable cells is of considerable interest. It would indicate that lethality from total body irradiation is not the result of bone marrow depletion alone, but due to damage to various organs, the bone marrow being actually more resistant to radiation than the body as a whole.

By the *in vitro* exposure of *homologous and heterologous bone marrow* to various dose levels of x-rays, it was hoped to remove host specificity and to abolish graft-versus-host reaction through x-ray induced mutation, chromosomal aberrations, breaks, deletion, and/or ploidy<sup>3</sup>. Obviously, several gene requirements would have to be altered in order that the foreign bone marrow be compatible with the new host<sup>4</sup>. It would appear that either *in vitro* irradiation did not render homologous and heterologous bone marrow immunologically incompetent, or that a 100% lethal dose might not adequately suppress the host's immunogenic system. Also, it would be conceivable to suspect that desoxyribonuclease played an important part in destruction of DNA that follows irradiation<sup>5</sup>. The inability of irradiated homologous and heterologous bone marrow to persist and proliferate, even temporarily in the new host, similar to isologous bone marrow, or to stimulate regeneration of the host's bone marrow was presumably because of the degree of genetic dissimilarity.

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*The Children's Cancer Research Foundation and the Departments of Pathology, The Children's Medical Center and Harvard Medical School, Boston (Mass.), July 1, 1959.*

Zusammenfassung

Wurde *in vitro* röntgenbestrahltes Knochenmark (475 r, 950 r, 1425 r) in letal bestrahlte Mäuse (LD 100/11 = 950 r) injiziert, so ergab sich: Isologes Knochenmark, mit 950 r bestrahlt, besitzt noch beachtliche Schutzwirkung; bestrahltes homologes und heterologes Knochenmark war weniger oder ganz inaktiv.

<sup>3</sup> T. T. PUCK, Proc. nat. Acad. Sci., Wash. **44**, 722 (1958).  
<sup>4</sup> N. A. MITCHISON, *Biological Replication of Macromolecules*, S. E. B. Symposia (Acad. Press. Inc., New York 1958), p. 225.  
<sup>5</sup> F. M. BACQ, P. FISCHER, A. HERVE, C. LIEBECQ, and S. LIEBECQ-HUTTER, Nature **182**, 175 (1958).

Immediate Effects of UV Irradiation on the Isolated Rat Blastocyst

It has recently<sup>1,2</sup> been reported that the inner cell mass of a rat blastocyst (4½ day after copulation) is destroyed after 1500 ergs/mm<sup>2</sup> of UV irradiation. By the tenth day of pregnancy, only cells descending from the trophoblast are found in a normally configured egg-chamber.

The present study is an attempt to elucidate these results, analyzing the immediate cytological changes after irradiation. We tried to investigate the following possible cytological effects:

(1) since it is well known<sup>3</sup> that the UV irradiation can retard or inhibit cell division and cleavage, we counted mitoses and cells of the blastocysts.

(2) The inner cell mass of a normal mammalian blastocyst shows a pronounced affinity for pyronine<sup>4</sup>. Such cytoplasmic basophilia may disappear after UV irradiation as has been shown in nucleate and anucleate fragments of ameba<sup>5</sup>.

(3) The pycnotic cells are often found after irradiation and the affinity of the nuclei for methyl green may disappear<sup>7</sup>.

As before, we used the modified egg-transfer method together with irradiation. Owing to this technique, the transplanted eggs are always in the right uterine horn and those of the recipient in the left one.

In order to test these possibilities, we recovered the irradiated and transplanted blastocysts from the uterine horn of the recipient after 6 (Series 1) or 17 h following irradiation (Series 2). The eggs were fixed *in toto*<sup>4</sup> and stained by the usual mixture after Unna-Brachet.

*Series 1.* The eggs recovered 6 h after irradiation (i. e. still the fifth day of pregnancy) showed no changes at all, if 1500 ergs/mm<sup>2</sup> were used, but they had some rare pycnotic cells after 3000 ergs/mm<sup>2</sup>. The average number of cells after both doses was 31.77 ± 1.19. Of 6 blastocysts (3000 ergs/mm<sup>2</sup>) in three there were 4 pycnotic cells. Controls had the same number of cells (33 ± 2.93), but no pycnotic cells. A total number of 17 blastocysts with 550 cells has been counted.

*Series 2.* After 16-17 or 19 h (i. e. already the sixth day of pregnancy) the average number of cells was as follows (dose 1500 ergs/mm<sup>2</sup>):

| Experiment (right uterine horn)   |                        | Controls (left uterine horn) |               |
|-----------------------------------|------------------------|------------------------------|---------------|
| a transplantation and irradiation | b transplantation only | b                            | a             |
| (8) 26 ± 1.57*                    | (7) 48.43 ± 1.98       | (5) 51 ± 4.09                | (7) 49 ± 0.03 |

\* = standard error. ( ) = number of blastocyst.  
Total number: 27 blastocysts with 1146 cells.

A considerable number of blastocysts were transplanted and irradiated, but only a small number was recovered and successfully fixed and stained.

Thus we may conclude that transplantation does not disturb the mitotic process, the number of cells being approximately the same as in the controls. Only the irradiated blastocysts have a smaller number of cells. The difference is highly significant (*P* < 0.01). This number (26) is not very different from the number of cells from series 1, recovered the fifth day of pregnancy (the difference is not significant).

The relative number of cells of the inner cell mass is approximately the same in irradiated blastocysts as in the controls (about 32-37%).

<sup>1</sup> N. ŠKREB and M. MÜLLER Proc. XV<sup>th</sup> Intern. Congr. Zool. London p. 1041 (1959).  
<sup>2</sup> N. ŠKREB and M. MÜLLER Naturwiss. **46**, 455 (1959).  
<sup>3</sup> R. F. KIMBALL, in A. HOLLANDER, *Radiation Biology*, vol. II (McGraw-Hill Co. 1955, p. 285).  
<sup>4</sup> A. DALCQ, Bull. Acad. R. med. Belg. **18**, VI. Sér., 236 (1952).  
<sup>5</sup> Y. ŠKREB and M. ERRERA, Exp. Cell Res. **12**, 649 (1957).  
<sup>6</sup> S. P. HICKS, Arch. Pathol. **55**, 302 (1953).  
<sup>7</sup> M. ERRERA, Biochem. biophys. Acta **7**, 665 (1951).  
<sup>8</sup> N. ŠKREB, B. LEVAK, M. MÜLLER, and G. LUKOVIC, Naturwiss. **45**, 451 (1958).  
<sup>9</sup> D. BODENSTEIN, J. cell. comp. Physiol. **43**, Suppl. 1, 179 (1954).



Mitoses were found rather rarely both in transplanted eggs and in controls, so that no conclusions may be drawn.

Pycnotic cells were observed only in irradiated blastocysts. All pycnotic cells were stained deeply green after Unna-Brachet, and were Feulgen positive. ERRERA<sup>7</sup>, using stronger doses than we did, has found that the affinity of the nuclei for methyl-green is weak after UV irradiation, but BODENSTEIN's<sup>9</sup> observations following treatment with mustard gas were the same as ours.

Out of 8 blastocysts 6 had 34 pycnotic cells of a total number of 208 cells, i. e. 16.35%.

As regards their position, in the majority of blastocysts the pycnotic cells were observed between the cells of inner cell mass and relatively rarely in the trophoblast. The difference is significant ( $X^2 = 4.825$ ,  $P < 0.05$ ).

The cytoplasm was normally stained with pyronine.

It can be concluded that the effect of UV irradiation (1500 ergs/mm<sup>2</sup>) may be observed about 17 h after irradiation: (1) the average number of the cells of a blastocyst is smaller than in controls, which may be explained by an inhibition of the mitotic process in the interphase of all cells,

(2) the pycnotic cells are readily visible and stain with methyl-green, especially between the cells of the inner cell mass.

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### Résumé

Sur le blastocyste du rat, irradié au cours de la transplantation par les rayons U. V. à la dose de 1500 ergs/mm<sup>2</sup>, on a pu observer précédemment que le développement du bouton embryonnaire est bloqué, tandis que le trophoblaste continue à se développer.

Dans cette note, nous avons voulu mettre en évidence les effets immédiats de cette irradiation:

Bien que les affinités du cytoplasme et du noyau pour le mélange vert de méthyle-pyronine restent inchangées, les cellules ne continuent pas à se diviser. Les noyaux pycnotiques sont relativement plus fréquents dans le bouton embryonnaire que dans le trophoblaste.

### «Bindung» eines synthetischen Polypeptides (Hypertensin) an Heparin und Freisetzung des Peptides durch Compound 48/80 *in vitro*

Der in den Mastzellen angereicherte Polysaccharid-schwefelsäureester Heparin liegt in diesen wohl vornehmlich als Histamin-heparinat vor<sup>1,2</sup>. Derart gebundenes Histamin kann sowohl *in vitro* wie *in vivo* durch verschiedene sogenannte Histamin-Liberatoren wie Compound 48/80<sup>3,4</sup> freigesetzt werden. WERLE und AMANN<sup>1</sup> konnten anhand von Dialyseversuchen zeigen, dass in Gemischen von Histamindihydrochlorid und Heparin (in Form des Na-Salzes) eine Bindung stattfindet mit Optimum im sauren Milieu (1%ige Essigsäure). Histamin lässt sich aus dieser Bindung durch Di- und Polyamine verdrängen. Da höhere Amine zu den wirksamsten Histamin-Liberatoren gehören<sup>1,5</sup>, lag es nahe zu untersuchen, ob Heparin auch Peptide zu binden vermag, und ob solche «gebundene» Peptide durch einen Histamin-Liberator wie Compound 48/80 freigesetzt werden können. Unsere Versuche befassten sich zur Hauptsache mit einem synthetischen Oktapeptid, Hypertensin, dessen Synthese und Pharmakologie von SCHWYZER *et al.*<sup>6</sup> sowie MEIER *et al.*<sup>7</sup>

beschrieben wurden. Im Gegensatz zu der Versuchsanordnung von WERLE und AMANN wurde nicht handelsübliches Heparin, sondern Heparinsäure verwendet, welche durch Behandlung von Handelsheparin (Hoffmann-La Roche) mit einem Ionenaustauscher (Amberlite J. R. 120 in der H<sup>+</sup>-Form) gewonnen wurde<sup>8</sup>. Auf diese Weise erhaltene Heparinsäure besass die gleichen physikalisch-chemischen Eigenschaften, wie sie von WILANDER für ein mittels Elektrodialyse gewonnenes Präparat beschrieben wurden<sup>9</sup>. Die Bindungsfähigkeit eines solchen Heparinsäurepräparates gegenüber Histamin verhielt sich im übrigen etwa wie diejenige von Na-Heparinat in essigsaurem Milieu; ebenso konnte Histamin durch Compound 48/80 aus dieser Bindung freigesetzt werden. Der Nachweis der aufgetretenen Bindung zwischen Heparinsäure und Hypertensin sowie der Freisetzung wurde wie folgt erbracht: 4 mg Hypertensin (als essigsäures Salz) wurden in 6 ml destilliertem Wasser gelöst und bei 20°C gegen 194 ml destilliertes Wasser dialysiert (Dialyse-Schüttelverfahren). Nach beendeter Dialyse wurde der Hypertensin-gehalt des Aussendialysats an drei verschiedenen Testobjekten (Blutdruck des Huhns, isolierter Rattenuterus und isoliertes Meerschweinchen-Ileum) ausgetestet. Es wurde zum Beispiel gefunden, dass nach 3 h im gesamten mindestens 0,5 mg Hypertensin ins Aussendialysat übergetreten waren. Hypertensin erwies sich demnach erwartungsgemäss als eine im Vergleich zu Histamin langsam dialysable Substanz. Aus einem Gemisch von 4 mg Hypertensin mit 7 mg Heparinsäure trat unter den gleichen Versuchsbedingungen weniger als 0,001 mg Hypertensin ins Aussendialysat über. Der Zusatz von 4 mg des Histamin-Liberators Compound 48/80 zu einem Gemisch von 4 mg Hypertensin und 7 mg Heparinsäure erhöhte den Durchtritt von Hypertensin deutlich (auf 0,24 mg in 3 h).

Obwohl unsere Befunde *in vitro* und ohne Berücksichtigung verschiedener physikalisch-chemischer Faktoren (pH, Diffusionsgeschwindigkeit, usw.) erhoben wurden, erscheint es nicht unwahrscheinlich, dass «Bindungs»-Reaktionen von der Art der hier beschriebenen eine prinzipielle physiologische Bedeutung besitzen. Jedenfalls weisen sie auf Vorgänge hin, die im Zusammenhang mit der physiologischen und pathogenetischen Funktion von Peptiden sowie homöostatischen Regulationsprozessen unter Beteiligung saurer Polysaccharide eine Rolle spielen könnten.

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Pharmazeutische Abteilung, Basel, 11. Januar 1960.

### Summary

It is shown that a synthetic octapeptide, hypertensin, can be bound to heparin *in vitro* in a fashion similar to that of histamine. Hypertensin can be liberated from this 'complex' by Compound 48/80.

<sup>1</sup> E. WERLE und R. AMANN, *Naturwissenschaften* 42, 583 (1955); *Klin. Wschr.* 34, 624 (1956).

<sup>2</sup> Editorial: *Brit. med. J.* 2, 219 (1956).

<sup>3</sup> Herrn Dr. A. C. WHITE, Wellcome Research Laboratories, Beckenham (England), danken wir auch an dieser Stelle für die Überlassung einer Versuchsmenge.

<sup>4</sup> W. FELDBERG und W. D. M. PATON, *J. Physiol.* 114, 490 (1951).

<sup>5</sup> F. C. MCINTOSH und W. D. M. PATON, *J. Physiol.* 109, 190 (1949).

<sup>6</sup> W. RITTEL *et al.*, *Helv. chim. Acta* 40, 614 (1957); *Angew. Chemie* 69, 179 (1957).

<sup>7</sup> R. MEIER *et al.*, *Exper.* 13, 361 (1957).

<sup>8</sup> Herrn Dr. G. HUBER sind wir für die Herstellung von Heparinsäure zu Dank verpflichtet.

<sup>9</sup> O. WILANDER, *Skand. Arch. Physiol.* 81, Suppl. 15 (1939).



### Decrease of Bacteriotropic Activity of Isoniazid on *M. tuberculosis* in the Presence of Anthranilic Acid

As shown by BÖNICKE<sup>1</sup>, *o*-aminobenzoic acid (anthranilic acid, OABA) has no influence on the activity of isoniazid on *M. phlei*. We have, in our own experiments<sup>2</sup>, reached the same conclusion with the saprophytic *Mycobacterium* ATCC 607. It was now of interest to find out whether this is true also in the case of virulent mycobacteria.

We attempted at first to exclude the possibility of physicochemical interactions between isoniazid and OABA, which could have resulted in the influence of OABA on the speed of isoniazid decomposition, even in the absence of mycobacteria in the medium. The decrease of isoniazid in concentration 20.0 µg/ml in Youmans' liquid medium, with or without 1% human albumine, and in the presence or absence of 50.0 µg/ml OABA, at 37°C, was followed by means of the method for isoniazid determination of WOLLENBERG<sup>3</sup>. Results are summarized in Table I.

It may be seen that OABA in the tested concentrations has no influence on the speed of isoniazid decomposition in Youmans' medium, either with or without human albumine. Therefore, further results might be attributed mainly to the interference of OABA with the mechanism of isoniazid action on *M. tuberculosis*.

The possible synergistic or antagonistic influence of OABA on the isoniazid activity was traced in the liquid Youmans' medium without albumine. It was distributed in 50 ml amounts in 250 ml Erlenmeyer flasks and seeded with one loopfull of a 15-days' old Youmans' surface culture of H<sub>37</sub>Ra strain of *M. tuberculosis*<sup>4</sup>. After 21 days incubation at 37°C all flasks were autoclaved, the bacterial mass separated by filtration on Schleicher-Schüll paper discs No. 589<sup>2</sup> and transferred from filter discs into Kjeldahl flasks. Then total nitrogen of the bacterial mass was determined by the usual Kjeldahl method. Isoniazid (Nicetal-Wander) and OABA (Lachema-Czechoslovakia) concentrations, as well as results of this experiment, may be found in Table II.

Later another experiment was started on Youmans' liquid medium with 1% human albumine. The medium was distributed in 5.0 ml amounts in tubes, which were then seeded with 0.05 ml of a 10 days old Dubos culture of the H<sub>37</sub>Rv strain of *M. tuberculosis*<sup>4</sup>. Results were read after 14 days' incubation at 37°C. The content of tubes was transferred into Kjeldahl flasks and total nitrogen determination of the grown bacterial mass was performed as mentioned above. Isoniazid and OABA concentrations, as well as results of this experiment, are to be seen in Table III.

Results of both experiments were analysed statistically with use of the F-test. Thus it was found that with the H<sub>37</sub>Ra strain OABA in concentration of 10.0 µg/ml antagonised significantly the bacteriotropic activity of isoniazid in 99.5% confidence limits. With the H<sub>37</sub>Rv strain and the same OABA concentration, differences between the activity of isoniazid alone and isoniazid with OABA were within the 95.0% confidence limit and this result is therefore not significant. However, by increasing the OABA concentration from 10.0 to 25.0 or 50.0 µg/ml, significant results were obtained in the 97.5 or 99.5% confidence limits respectively.

The decrease of bacteriotropic potencies of isoniazid in the presence of OABA is very interesting in view of the exactly opposite activity of *p*-aminobenzoic acid,

Table I

| Medium                           | Days | Isoniazid* | Isoniazid + OABA* |
|----------------------------------|------|------------|-------------------|
| Youmans . . . . .                | 0    | 100        | 100               |
|                                  | 1    | 81         | 84                |
|                                  | 3    | 52         | 55                |
|                                  | 5    | 36         | 35                |
|                                  | 9    | 23         | 22                |
| Youmans . . . . .<br>1% albumine | 0    | 100        | 100               |
|                                  | 1    | 58         | 50                |
|                                  | 3    | 32         | 29                |
|                                  | 5    | 24         | 19                |
|                                  | 9    | 16         | 13                |

\* Isoniazid concentration in % terms; 100% equals to 20.0 µg/ml; OABA concentrations 50 µg/ml

Table II

| Drug                | mg of N | Statistical significance                            |
|---------------------|---------|-----------------------------------------------------|
| Isoniazid . . . . . | 0.80    | 91.54    31.33<br>31.33 = F <sub>1,4</sub> (0.005)* |
| 0.05 µg/ml          | 0.80    |                                                     |
|                     | 0.59    |                                                     |
| Isoniazid . . . . . | 2.24    |                                                     |
| 0.05 µg/ml          | 2.38    |                                                     |
| OABA                |         |                                                     |
| 10.0 µg/ml          | 2.80    |                                                     |

\* 0.5% critical value of F<sub>1,4</sub>-distribution

Table III

| Drug            | mg of N | F-Test                                                | Statistical significance for comparison with isoniazid alone |
|-----------------|---------|-------------------------------------------------------|--------------------------------------------------------------|
| Isoniazid . . . | 4.35    | 6.26    7.71<br>7.71 = F <sub>1,4</sub><br>(0.05)     | not found                                                    |
| 0.1 µg/ml       | 4.88    |                                                       |                                                              |
|                 | 4.08    |                                                       |                                                              |
| Isoniazid . . . | 4.35    | 19.69    12.22<br>12.22 = F <sub>1,4</sub><br>(0.025) | 2.5% significance level                                      |
| 0.1 µg/ml       | 4.88    |                                                       |                                                              |
| OABA 10.0 µg/ml | 4.61    |                                                       |                                                              |
| Isoniazid . . . | 5.02    | 38.53    34.12<br>34.12 = F <sub>1,3</sub><br>(0.01)  | 1% significance level                                        |
| 0.1 µg/ml       | 4.62    |                                                       |                                                              |
| OABA 25.0 µg/ml | 5.02    |                                                       |                                                              |
| Isoniazid . . . | 4.88    |                                                       |                                                              |
| 0.1 µg/ml       | 5.02    |                                                       |                                                              |
| OABA 50.0 µg/ml |         |                                                       |                                                              |

<sup>1</sup> R. BÖNICKE, Naturwissenschaften 39, 402 (1952).

<sup>2</sup> R. URBANČÍK, Amer. Rev. Tuberc. 78, 802 (1958).

<sup>3</sup> O. WOLLENBERG, Klin. Wschr. 30, 906 (1952).

<sup>4</sup> Kindly supplied by Dr. W. STEENKEN from Trudeau Laboratories.

<sup>5</sup> E. KRÜGER-THIEMER, Amer. Rev. Tuberc. 77, 364 (1958).



which increases the activity of isoniazid on *M. tuberculosis in vitro*<sup>2</sup>. The mechanism by which OABA is metabolized in mycobacteria remains entirely unknown. We may state only that in tested concentrations OABA itself does not interfere with the growth of either strain used. It might be suggested that it is somehow connected with the metabolism of nicotinic acid. The latter is now supposed to be the point that is attacked by isoniazid, as suggested by KRÜGER-THIEMER<sup>5</sup>. However an explanation of the facts presented cannot be given before we know the precise mechanism of isoniazid activity against *M. tuberculosis*.

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Zusammenfassung

Anthranilsäure antagonisiert die Isoniazid-Aktivität gegen *M. tuberculosis in vitro*. Dieser Antagonismus beruht auf keinem physikalisch-chemischen Einfluss der Anthranilsäure auf die Geschwindigkeit der Isoniazid-Spaltung im flüssigen Nährboden.

Inhibition of Sea Urchin Egg Cleavage by Ribonuclease<sup>1</sup>

I. *Lytechinus variegatus* - *Arbacia punctulata*

In view of the demonstrated inhibitory action of ribonuclease (RNase) on cell division in amphibian eggs<sup>2,3</sup> and certain other cells<sup>4</sup>, it seemed of interest to examine for similar action of ribonuclease on cleavage of sea urchin eggs. This material is of special interest because of the demonstration by MAZIA of ribonucleic acid in the isolated mitotic apparatus of sea urchin eggs<sup>5</sup> and the report of ribonucleic acid in the cell surface of *Arbacia* eggs by LANSING and ROSENTHAL<sup>6</sup>. Because of the differences in biological activity found in different ribonuclease preparations<sup>7</sup>, several crystalline enzyme samples were used. They were obtained from the following commercial sources: Armour, Sigma, Worthington, and Nutritional Biochemical Corporation. These enzymes were tested for action on fertilized eggs of two species of sea urchins, *Lytechinus variegatus* and *Arbacia punctulata*. Eggs were added to sea water solutions of the enzyme preparations at selected intervals following fertilization and before second cleavage. They were subsequently examined for cleavage without removal from the RNase solutions. Appropriate concentration of all four enzyme preparations (M. W. = 14,000) inhibited cleavage of both species. However, as the Table shows, the several preparations are not equally effective. Similar results were obtained in eight other experiments. This variation in activity may be due to differing proportions of acid and alkaline ribonuclease in the several preparations<sup>8</sup>. Several other enzymes did not affect cleavage of *Arbacia* eggs. These include trypsin (see also <sup>9</sup>), lysozyme, and deoxyribonuclease. Aside from an inhibiting action on cleavage, the enzyme preparations had other effects. These include a collapse and partial lysis of the fully formed *Lytechinus* fertilization membrane (this effect was not pronounced in *Arbacia*) and an egg jelly precipitating action by all four enzyme preparations. However, these effects are not related to the inhibition of cleavage by RNase because eggs from which fertilization membranes had been removed were also inhibited by RNase. Such demembrated *Lytechinus* eggs increased (without lysis) 25% in

diameter upon standing in the same ribonuclease. This swelling suggests action on the cellular membrane. The almost complete inhibition of the cleavage of sea urchin eggs reported here has been obtained with eight different batches of eggs. In five other experiments performed independently by one of us (C. B. M.) an inhibition was obtained (with Armour and Worthington RNase at the concentration level of  $4 \cdot 10^{-4}$  M) but not such a pronounced one. In this case, merely a retardation of the cleavage was obtained (RNase-treated are two to three divisions behind controls) followed by abnormal development sometimes resulting in death of the embryo. This could indicate that the permeability to RNase might be very dependent on the actual condition of the cellular membrane in the case of this material. On the other hand, preliminary observations on *Lytechinus* suggest an increased ribonuclease sensitive period, 20 to 40 min before cell division. Such a critical stage, also observed by RUSTAD for U.V. sensitivity<sup>5</sup>, may be found to be correlated with a specific action of ribonuclease on some part of the mitotic apparatus.

| Source of Ribonuclease  | $3 \times 10^{-4}$ | Concentration          |                      |
|-------------------------|--------------------|------------------------|----------------------|
|                         |                    | $1.5 \times 10^{-4}$ M | $7 \times 10^{-5}$ M |
| Nutritional Biochemical | 1                  | 1-2                    | 2-4                  |
| Armour . . . . .        | 1                  | 2-4                    | 2-8                  |
| Sigma . . . . .         | 1                  | 2-8                    | Young morula         |
| Worthington . . . . .   | 1                  | Young morula           | Young morula         |

Cell stage at which *Arbacia* egg cleavage was blocked by three concentrations of the preparations, in conditions where complete inhibition was achieved (controls are at the stage young morulas)

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Résumé

La ribonucléase exerce une action inhibitrice sur le clivage des œufs d'oursins. La source commerciale de l'enzyme, la concentration utilisée et l'état des œufs déterminent les effets obtenus. Ceux-ci varient entre une inhibition totale immédiate et un ralentissement des divisions cellulaires, suivi d'un développement anormal éventuellement abortif. En outre, la ribonucléase a une action complexe sur la membrane de fertilisation, la couche hyaline et la membrane cellulaire. Cette dernière action pourrait être liée aux effets antimitotiques observés.

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<sup>1</sup> Contribution No.126 from the Oceanographic Institute, Florida State University, Tallahassee (Florida). Aided by a grant from the National Science Foundation.  
<sup>2</sup> L. LEDOUX, J. LeCLERC, and F. VANDERHAEGHE, *Nature* 174, 793 (1954).  
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<sup>4</sup> J. BRACHET, *Biochemical Cytology* (Academic Press, New York 1957), p. 157.  
<sup>5</sup> D. MAZIA, *Adv. biol. med. Phys.* IV (1956). – R. C. RUSTAD, *Exp. Cell Res.* 16, 575 (1959) and in press.  
<sup>6</sup> A. I. LANSING and T. B. ROSENTHAL, *Biol. Bull.* 97, 263 (1949).  
<sup>7</sup> L. LEDOUX and J. BRACHET, *Biochem. biophys. Acta* 16, 290 (1955).  
<sup>8</sup> L. LEDOUX, unpublished.  
<sup>9</sup> A. TYLER and C.B. METZ, *Pubbl. Staz. zool. Napoli* 27, 128 (1955).



## Induced Radiation Sensitivity with Four Radiation Protective Chemicals<sup>1</sup>

Many factors have been found to influence the dose-survival response of various forms of life. Among the factors studied has been the pretreatment of microorganisms and other cellular forms with various chemicals which induce an increased resistance to subsequent irradiation. Such radiation protection has been reviewed by HOLLAENDER and STAPLETON<sup>2</sup>, PATT<sup>3</sup>, STAPLETON<sup>4</sup>, and others. In the course of several investigations, we have had occasion to use several of these protective chemicals on microorganisms and have encountered discrepant results from those previously reported.

The microorganisms used were *Nocardia corallina* (ATCC 4273), *Staphylococcus aureus* (O. U. culture collection), and *Escherichia coli* B/r (O. U. culture collection). *N. corallina* was grown on nutrient agar containing 1% fructose and incubated for 4 days at 29°C. *Staph. aureus* and *E. coli* B/r were grown on nutrient agar and incubated for 2 days at 37°C. Cell suspensions were prepared in saline blanks which were shaken on a vibratory shaker for 5 min and then centrifuged for 4 min at 2000 *g* to remove cell clumps. Each final suspension was examined microscopically for excessive clumping and was discarded unless it contained at least 90% single cells.

The chemicals used were C. P. glycine, pyruvic acid, sodium hydrosulfite, and phenol. Each chemical was prepared in a one molar concentration within one week prior to final dilution. The final concentration for each chemical, as determined by tolerance tests, was found to be glycine, 0.01 *M*; pyruvic acid, 0.1 *M*; sodium hydrosulfite, 0.1 *M*; and phenol, 0.01 *M*. One ml inoculum of each cell suspension was added to 5 ml of each chemical, and after 15 min contact, the organisms were centrifuged, washed, and resuspended in sterile physiological saline. The resulting suspensions were then irradiated with either X-ray or ultraviolet light.

X-irradiation was obtained from a Picker portable field unit operating at 96 kv and 4 ma using aluminium filtration. At the target distance used, the dose rate was 1000 r/min as measured with a Victoreen R meter. Cell suspensions were X-irradiated for varying times, appropriately diluted, and plated in nutrient agar. Ultraviolet irradiation was obtained from a Will Corporation germicidal lamp at a target distance of 7 inches for *N. corallina* and 14 inches for *Staph. aureus* and *E. coli* B/r. Surface plated suspensions in appropriate dilution were irradiated with the ultraviolet light.

The protective effect of pretreatment of *E. coli* B/r with the four chemicals was confirmed with both X-ray and ultraviolet light. However opposite results were obtained with *S. aureus* and *N. corallina*. Instead of displaying a protective effect, each of the four chemicals sensitized *S. aureus* to X-irradiation as shown in Figure 1. Glycine was found to have the smallest sensitization effect and pyruvic acid caused the greatest sensitization. The effect of the chemicals on the ultraviolet dose-survivor response of the organisms is given in Figure 2. With both sodium hydrosulfite and phenol treated cultures, there was no appreciable change from the rate of inactivation of the untreated culture. However both pyruvic acid and glycine sensitized *S. aureus* to ultraviolet light. Similar results using *N. corallina* were obtained with X-irradiation (Figure 3) and ultraviolet light (Figure 4).

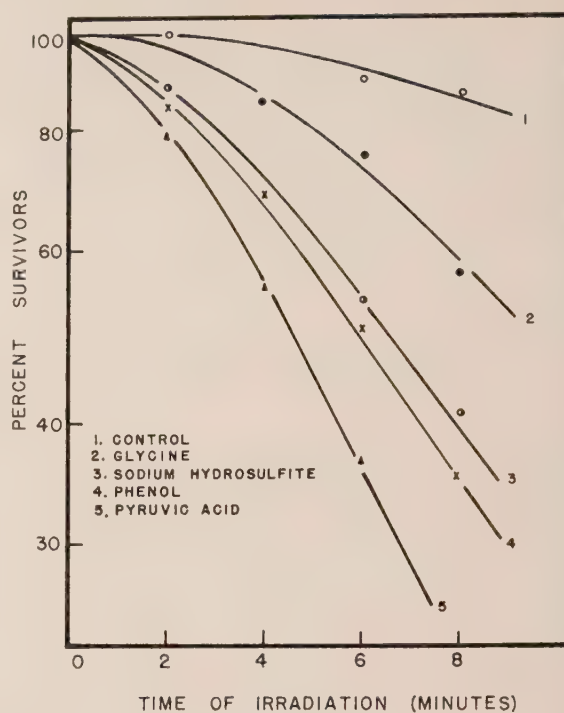


Fig. 1.—The effect of four 'protective' chemicals on the X-ray response of *Staphylococcus aureus*

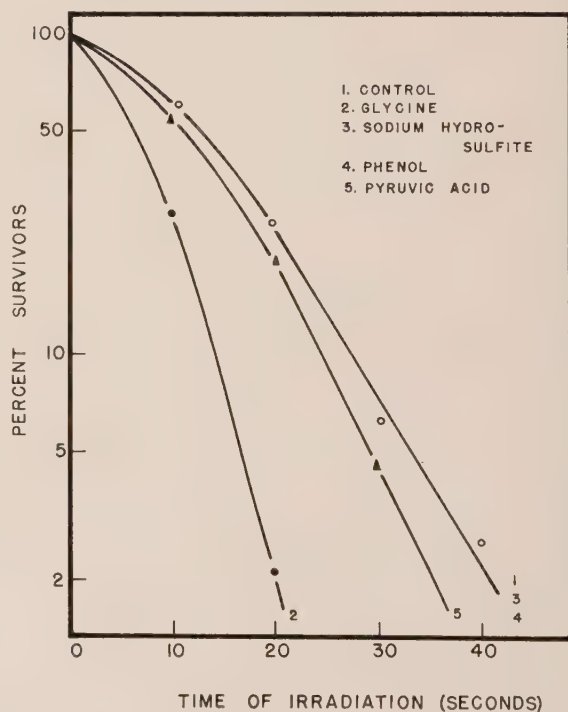


Fig. 2.—The effect of four 'protective' chemicals on the ultraviolet response of *Staphylococcus aureus*

<sup>1</sup> This work was taken in part from a thesis submitted by the senior author to the Graduate Faculty of the University of Oklahoma in partial fulfillment of the requirements for the M. S. degree. The work was supported in part by a grant from the Atomic Energy Commission under contract No. AT(40-1)-1976.

<sup>2</sup> A. HOLLAENDER and G. E. STAPLETON, *Physiol. Rev.* 33, 77 (1953).

<sup>3</sup> H. M. PATT, *Physiol. Rev.* 33, 35 (1953).

<sup>4</sup> G. E. STAPLETON, *Ann. N. Y. Acad. Sci.* 59, 604 (1955).



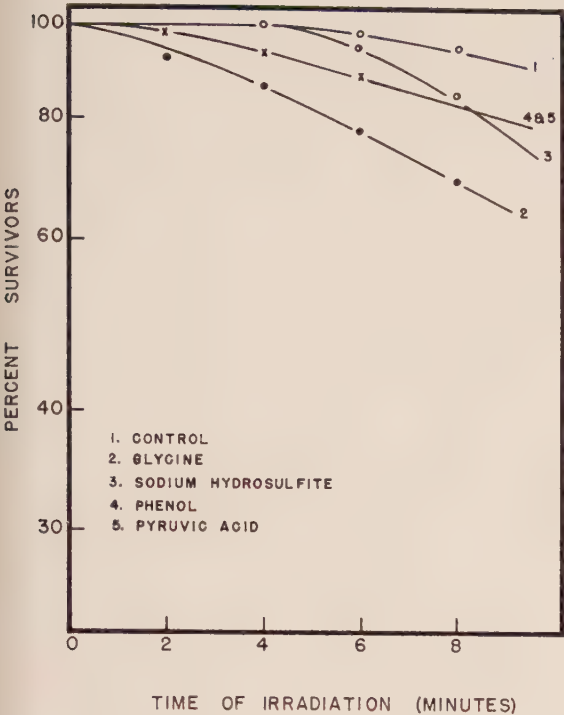


Fig. 3.—The effect of four 'protective' chemicals on the X-ray response of *Nocardia corallina*

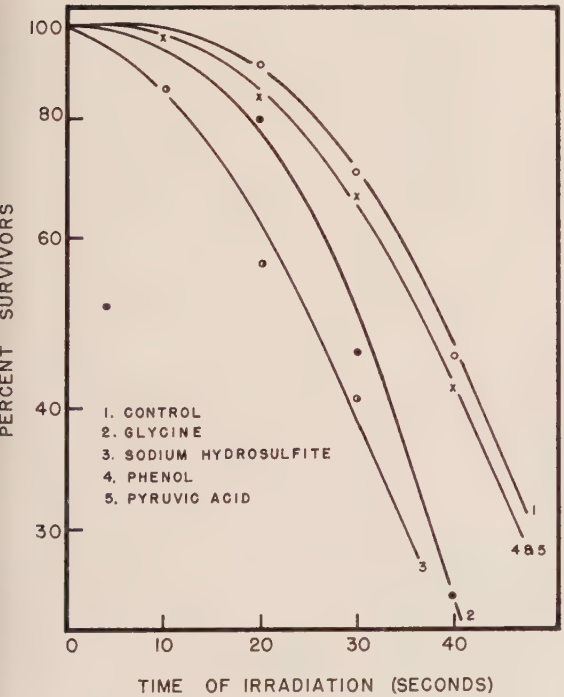


Fig. 4.—The effect of four 'protective' chemicals on the ultraviolet response of *Nocardia corallina*

According to PATT<sup>3</sup>, the radiation protective effect of pretreatment of cell suspensions with various chemicals supports the premise that many of the biological effects of radiation are indirect. This mainly involves the transfer of energy from radiation activated water molecules to essential biological components. Many of the protective effects could thus be explained on the basis of both the treatment chemical and some biological component competing for active radicals which are formed in the irradiated water. However such simple competition cannot be used as an explanation in all cases, and therefore more complex energy transfer schemes have been postulated. It does not appear probable that competition for active radicals should be operative in *E. coli* B/r and not in *S. aureus* and *N. corallina*.

STAPLETON<sup>4</sup> suggested that the lethal action of ionizing radiations on bacterial cells could be due to a radiation initiated chain reaction that results in the ultimate destruction of a large number of biologically important molecules. The terminal result of the chain reaction in the case reported here would be the inhibition of cell division, which is the cellular function which was used as an index of radiation effect. The inhibition of cell division could be caused by any one of many cellular alterations, so no single terminal action of the radiation was detected.

There is accumulated evidence that some radiation damage has a delayed effect, and some radiation damage may be repaired by the cell<sup>5,6</sup>. Since a so-called protective chemical may impart increased radiation resistance to one genus and radiation sensitivity in another, it is conceivable that the chemical alters the normal metabolism of the cell to such an extent as to enhance or impair the radiation recovery ability of the cell. If such is the case, it would appear that a study of the protective action of various chemicals on cells of different metabolic types might yield better insight into the mechanisms involved in the protective action.

A second explanation of the discrepant results reported here is that an oxygen effect is the determining cause. Each of the cells studied may have different internal availability of oxygen which is altered in the chemically changed metabolic pattern of the cell. If so, again the study of metabolic types could aid in a better understanding of protective action.

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Department of Plant Sciences, University of Oklahoma, Norman, October 10, 1959.

Résumé

L'action protectrice de la glycine, de l'acide pyruvique, du phénol, et de la soude hydrosulfite contre les dommages causés à l'*Escherichia coli* par l'irradiation aux rayons X ou bien aux rayons ultraviolets a été confirmée. Toutefois, il a été constaté que ces corps chimiques rendent les cellules de *Staphylococcus aureus* et de *Nocardia corallina* plus sensibles à l'action de ces deux radiations.

<sup>5</sup> A. HOLLAENDER (Ed.), *Radiation Biology*, Vol. I. *High Energy Radiation* (McGraw Hill, New York).

<sup>6</sup> A. HOLLAENDER (Ed.), *Radiation Biology*, Vol. II. *Ultraviolet and Related Radiations* (McGraw Hill, New York).



## Ion Adsorption and Excitation

### III.

It has been demonstrated that the ions involved in the fluxes characteristic of the excitation of squid giant axons form a monolayer at the functional nerve surface and that they are not hydrated<sup>1</sup>. As a consequence it was postulated that the resting potential of these nerves can be regarded as the algebraic sum of the potentials as calculated separately for each of the major monovalent ions represented at the surface monolayer, the assumption proving consistent with the data found in the literature<sup>2</sup>.

The resting potential appears thus to express solely the ionic asymmetry obtaining between the nerve surface monolayer and the outer solution. The use of the gross internal ionic concentration in the calculation of the resting potential appears to be justified because the monovalent ion composition of the surface monolayer is identical with that of the nerve interior. This identity arises from the definition of the volume  $\Delta v$  which at the functional nerve surface represents one half of the volume of a monolayer of a given ionic species<sup>1</sup>.

The rapidity with which the nerve regains its ability to conduct a second impulse is apparently contradicted by the demonstration that recovery is a prolonged process, the thermal events following a single impulse lasting some 300 ms<sup>3</sup>. Since the nerve surface monolayer acquires the ionic composition characteristic of the outer solution as a consequence of the passage of an impulse<sup>1</sup>, the paradox can be resolved if it is assumed that the return of the potential to its resting value is due to the establishment of a new interface some 17 Å beneath the former one. The value of 17 Å is taken since it represents the average distance between the  $K$  ions in the axoplasm. This new interface ought to appear concomitantly with the cessation of the ionic equilibrations accompanying the previous impulse, the velocity of its appearance being a function of the length of the diffusion path and velocity of the fluxing ions. A rapid train of impulses would thus cause the nerve to enter into a sort of 'debt' because of the creation of a peripheral plasma layer in which the monovalent ion atmosphere is identical with that of the outer solution in regard to composition.

Assuming the diffusion coefficient of ions in the outer neuroplasm to be identical with that in a solution of a comparable molality, the thickness of the peripheral layer of normal resting axon can be estimated from the diffusion formula<sup>4</sup>

$$C = C_0 \left( 1 - \frac{2}{\pi} \int_0^y e^{-y^2} dy \right) \text{ where } y = \frac{x}{2\sqrt{Dt}}$$

where  $x$  = thickness of the layer in cm

$$D = 1.534 \times 10^{-5} \text{ cm}^2/\text{s}$$

$$t = 2.45 \times 10^{-3} \text{ s (duration of the impulse as measured on an oscilloscope trace)}^5$$

$$C_0 = K_i = 345 \text{ mM/kg}$$

$$C = K_o = 22 \text{ mM/kg}$$

According to this calculation, the functional surface of a normal nerve, i. e. the interface across which the resting potential is manifested is situated 5  $\mu$  below the morphological surface.

The views expressed here are in a sense shared by SHANES<sup>6</sup> who considers such a peripheral layer to be 16  $\mu$  thick, this value resulting from tracer diffusion kinetics, and by SHAW<sup>7</sup> according to whom about 1/3 of the muscle fiber volume, in contrast to the rest of the cell, is regarded as being 'in physicochemical equilibrium' with the outer solution. HARRIS<sup>8</sup> likewise postulated the presence in frog muscle of a  $\text{Na}^+$  rich annular layer about 3  $\mu$  thick. In red cells this peripheral layer is also believed capable of expansion under the influence of strophanthidin<sup>9</sup>.

The presence of such a peripheral plasma layer in nerves would account not only for such physical effects as the 'fast' component of tracer exchange, but would also serve to interpret the 'tiring' of nerve preparations and the attendant lengthening of the spikes in terms of longer diffusion paths.

The deeply staining peripheral layer of neurons long known to appear as a consequence of stimulation<sup>10</sup> is assumed to be identical with the plasma layer postulated here. As a further likelihood, the molecular modifications occurring in stimulated nerves as revealed by various protein denaturation changes<sup>11-13</sup> may be considered as localized at the functional surface of the nerve. In fact, these structural rearrangements may well prove to be the primary cause of changes in both the ionic composition and stainability of the nerve periphery.

I wish to thank Dr. B. W. ZWEIFACH for his kind advice and interest.

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### Résumé

Les données expérimentales suggèrent que la surface fonctionnelle du nerf (c'est-à-dire la couche à travers laquelle le potentiel de repos se manifeste) est située à une certaine distance de la surface morphologique de l'axone et sa localisation à chaque moment dépend de l'état fonctionnel de la cellule. D'après un calcul fondé sur la durée du « spike » de l'axone du Calmar, la surface fonctionnelle de la cellule serait à 5  $\mu$  de la surface morphologique.

<sup>1</sup> E. ASCHHEIM, *Science* 129, 779 (1959).

<sup>2</sup> E. ASCHHEIM, *Exper.* 15, 440 (1959).

<sup>3</sup> B. C. ABBOTT, A. V. HILL, and J. HOWARTH, *Proc. Roy. Soc. B* 148, 149 (1958).

<sup>4</sup> T. SVEDBERG, *Colloid Chemistry* (Chemical Catalog Co., New York 1928).

<sup>5</sup> K. S. COLE and H. J. CURTIS, *J. gen. Physiol.* 24, 551 (1940).

<sup>6</sup> A. M. SHANES and M. D. BERMAN, *J. gen. Physiol.* 39, 279 (1955).

<sup>7</sup> S. E. SIMON, F. H. SHAW, S. BENNET, and M. MUELLER, *J. gen. Physiol.* 40, 753 (1956-1957).

<sup>8</sup> E. J. HARRIS, *J. gen. Physiol.* 41, 169 (1957).

<sup>9</sup> E. J. HARRIS and T. A. J. PRANKERD, *J. gen. Physiol.* 41, 197 (1957).

<sup>10</sup> R. FISCHER and W. ZEMAN, *Nature* 183, 1337 (1959).

<sup>11</sup> G. UNGAR, E. ASCHHEIM, and S. PSYCHOYOS, 20th Intern. Physiol. Congr. Brussels, 904 (1956).

<sup>12</sup> G. UNGAR, E. ASCHHEIM, S. PSYCHOYOS, and D. V. ROMANO, *J. gen. Physiol.* 40, 635 (1957).

<sup>13</sup> G. UNGAR, D. V. ROMANO, and E. ASCHHEIM, *Fed. Proc.* 16, 130 (1957).



**In vitro Inhibition of Anaerobic Glycolysis  
by Phenothiazine Ataractic Drugs**

Although a variety of enzymatic effects have been demonstrated with chlorpromazine and related phenothiazine derivatives, relatively little attention has been given to the effect of such compounds on anaerobic glycolysis. BERNSOHN, NAMAJUSKA, and COCHRANE reported that rat brain hexokinase activity was inhibited markedly by  $10^{-3}$  M chlorpromazine but that the anaerobic glycolytic activity of fortified rat brain homogenates was not altered by this rather high concentration of the drug<sup>1</sup>. More recently, MORACZEWSKI and DU BOIS found that the same concentration of chlorpromazine had no effect on anaerobic glycolysis in rat or mouse liver homogenates and that a single, massive dose of the drug did not reduce the glycolytic activity of mouse liver slices<sup>2</sup>. In addition, these workers demonstrated slight inhibition of glycolysis in rat and mouse liver homogenates by  $10^{-3}$  M chlorpromazine sulfoxide. Studies in this laboratory have shown that several phenothiazines, including chlorpromazine, are capable of inhibiting anaerobic glycolysis in rat brain homogenates provided that the incubation time is extended beyond the 30–40-min periods employed by BERNSOHN *et al.* and MORACZEWSKI and DU BOIS.

The results presented in the Table show that while  $10^{-5}$  M chlorpromazine has no effect on glycolysis in rat brain homogenates,  $10^{-3}$  M concentrations of the drug produce a striking effect after 1 h. Essentially identical results were observed with two other phenothiazine ataractic agents, promazine and mepazine. From a consideration of the structures of these three drugs it can be inferred that neither the 2-chloro substitution nor the N-(3'-dimethylaminopropyl) of chlorpromazine moiety is essential for inhibition of anaerobic glycolysis by phenothiazines.

Inhibition of Anaerobic Glycolysis in Rat Brain Homogenates by Chlorpromazine

| System                      | Glucose added | $Q_{CO_2}^{N_2}$ |                 |                 |
|-----------------------------|---------------|------------------|-----------------|-----------------|
|                             |               | 1 h              | 2 h             | 3 h             |
| Endogenous . .              | —             | $1.99 \pm 0.64$  | $0.99 \pm 0.41$ | $0.90 \pm 0.19$ |
| Control . . . .             | +             | $5.32 \pm 0.61$  | $3.48 \pm 0.83$ | $3.46 \pm 0.41$ |
| Chlorpromazine, $10^{-5}$ M | +             | 5.70             | 2.70            | 3.30            |
| Chlorpromazine, $10^{-3}$ M | +             | 4.60             | 1.66            | 1.70            |

This finding, together with the positive results reported by BERNSOHN *et al.*<sup>1</sup> and MORACZEWSKI and DU BOIS<sup>2</sup>, suggest that phenothiazines may be converted slowly under anaerobic conditions, more rapidly in the presence of oxygen, to metabolic products which are capable of inhibiting anaerobic glycolysis, possibly through altering the rate of the hexokinase reaction. The fact that high concentrations of chlorpromazine are required to bring about this effect could be ascribed to the quantitative aspects of this postulated metabolic pathway; or perhaps interpreted as being related to the toxic rather than the therapeutic mechanism of action of the drug.

*Conditions*

Normal, male albino rats were decapitated, the brains removed and the cerebral cortex homogenized immediately in a Potter-Elvehjem grinder in 4 parts of ice-cold

Krebs-Ringer bicarbonate buffer<sup>3</sup>. To the main compartment of standard Warburg flasks, containing 2.3 ml of buffer or a glucose-buffer solution, was added 0.7 ml of homogenate (about 33 mg dry weight). The compounds to be studied<sup>4</sup> were dissolved in the glucose-buffer solution and added to give a final concentration of  $10^{-5}$  M or  $10^{-3}$  M. Where present, the final concentration of glucose was 0.2%. All flasks were gassed with  $N_2$ - $CO_2$  (95%–5%) and equilibrated to 38°C over a 20-min period. Measurements of gas production were made at 15 min intervals and results expressed in terms of  $Q_{CO_2}^{N_2}$  ( $\mu$ l  $CO_2$ /mg dry weight/h) calculated over the indicated periods. Data reported are averages of 12 determinations  $\pm$  one standard deviation ('endogenous' and 'controls' systems) or averages of two experiments ('chlorpromazine' systems).

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*Zusammenfassung*

Im Gegensatz zu Feststellungen anderer Forscher hemmten Chlorpromazin und zwei strukturell verwandte ataraktisch wirkende Phenothiazinderivate die anaerobe Glykolyse von homogenisiertem Rattenhirn. Der Nachweis dieser Hemmung bedarf einer längeren Inkubation, was auf die Mitwirkung eines zeitbedingten Vorgangs, wie metabolische Umwandlung der Phenothiazine in Hemmstoffe, deutet.

<sup>1</sup> J. BERNSOHN, I. NAMAJUSKA, and L. S. G. COCHRANE, Arch. Biochem. Biophys. 62, 274 (1956).  
<sup>2</sup> A. S. MORACZEWSKI and K. P. DU BOIS, Arch. int. Pharmacodyn. 120, 201 (1959).  
<sup>3</sup> W. W. UMBREIT, R. H. BURIS, and J. F. STAUFFER, *Manometric Techniques and Tissue Metabolism* (2nd Edition, Burgess Publishing Co., Minneapolis 1949), p. 119.  
<sup>4</sup> Chlorpromazine was obtained from Smith, Kline and French Laboratories, Philadelphia, mepazine from Warner-Chilcott Laboratories, Morris Plains, New Jersey, and promazine from Wyeth Laboratories, Inc., Philadelphia.

**Aufhebung der Knospenruhe von Reben  
durch Rindite**

Die Knospenruhe der Reben erreicht in den Monaten September bis November ihre grösste Tiefe. Durch eine Kältebehandlung ist sie nicht zu verkürzen, während Gibberellinsäure je nach Sorte und Zeitpunkt der Behandlung austriebshemmend wirkt<sup>1</sup>. Es wurde darum versucht, die Knospenruhe durch Behandlung mit dem von DENNY<sup>2</sup> an Kartoffelknollen entwickelten, austriebsfördernden Gemisch von Äthylenchlorhydrin, Äthylen-dichlorid und Tetrachlorkohlenstoff (Volumenverhältnis 7:3:1), das als Rindite bekannt ist, zu brechen, da dieses Substanzgemisch bereits mehrfach erfolgreich zur Aufhebung der Samenruhe<sup>3,4</sup> und auch vereinzelt zur Förderung des Austriebes ruhender Winterknospen<sup>5</sup> verwendet wurde.

<sup>1</sup> G. ALLEWELDT, Naturwiss. 46, 434 (1959).  
<sup>2</sup> F. E. DENNY, Contr. Boyce Thompson Inst. 14, 1 (1945).  
<sup>3</sup> I. SZALAI, Acta agron. Acad. Sci. hungaricae 7, 25 (1957).  
<sup>4</sup> O. FISCHNISCH, A. GRAHL und M. THIELEBEIN, Landbauforsch. Völkenrode 8, 4 (1958).  
<sup>5</sup> S. THORUP, Physiol. Plantarum 10, 728 (1957).



Tabelle I. Die Wirkung von Rindite, Äthylenchlorhydrin und Äthylenchlorid auf den Austrieb der Winterknospen 1jähriger Reben (Zuchtstamm FS. 4-201-39)

| Versuchs-<br>beginn | Variante                     | Pflanzenzahl<br><i>n</i> | Tage bis zum Austrieb |         | ausgetriebene |     |          |    |
|---------------------|------------------------------|--------------------------|-----------------------|---------|---------------|-----|----------|----|
|                     |                              |                          | <i>x</i>              | $\pm m$ | Pflanzen      |     | Knospen  |    |
|                     |                              |                          |                       |         | $\Sigma$      | %   | $\Sigma$ | %  |
| 13. 9. 59           | unbehandelt . . . . .        | 20                       | 18,3                  | 1,59    | 4             | 20  | 6        | 7  |
|                     | Rindite . . . . .            | 10                       | 10,0                  | 0,01    | 10            | 100 | 28       | 70 |
| 28. 9. 59           | unbehandelt . . . . .        | 8                        | —                     | —       | —             | —   | —        | —  |
|                     | Rindite . . . . .            | 8                        | 12,1                  | 0,50    | 8             | 100 | 15       | 47 |
|                     | Äthylenchlorhydrin . . . . . | 8                        | 11,6                  | 0,34    | 8             | 100 | 16       | 50 |
|                     | Äthylenchlorid . . . . .     | 8                        | 27,0                  | —       | 4             | 50  | 4        | 13 |
| 23. 10. 59          | unbehandelt . . . . .        | 6                        | —                     | —       | —             | —   | —        | 50 |
|                     | Rindite . . . . .            | 12                       | 19,7                  | 0,23    | 12            | 100 | 24       | —  |

Tabelle II. Austriebsförderung von 1-Augenstecklingen durch Rindite

| Versuchs-<br>beginn | Sorte                    | Variante    | Pflanzenzahl<br><i>n</i> | Tage bis zum Austrieb |         | ausgetriebene<br>Knospen |    |
|---------------------|--------------------------|-------------|--------------------------|-----------------------|---------|--------------------------|----|
|                     |                          |             |                          | <i>x</i>              | $\pm m$ | $\Sigma$                 | %  |
| 17. 9. 59           | Sbl. 2-19-58 . . . . .   | unbehandelt | 34                       | —                     | —       | —                        | —  |
|                     |                          | Rindite     | 20                       | 12,2                  | 0,40    | 16                       | 80 |
| 5. 10. 59           | Sbl. 2-19-58 . . . . .   | unbehandelt | 31                       | —                     | —       | —                        | —  |
|                     |                          | Rindite     | 33                       | 18,7                  | 0,33    | 24                       | 73 |
| 15. 10. 59          | Sbl. 2-19-58 . . . . .   | unbehandelt | 25                       | —                     | —       | —                        | —  |
|                     |                          | Rindite     | 20                       | 23,2                  | 1,62    | 18                       | 90 |
| 5. 10. 59           | Sylvaner . . . . .       | unbehandelt | 17                       | —                     | —       | —                        | —  |
|                     |                          | Rindite     | 27                       | 16,0                  | 0,35    | 24                       | 89 |
| 15. 10. 59          | Sylvaner . . . . .       | unbehandelt | 25                       | —                     | —       | —                        | —  |
|                     |                          | Rindite     | 22                       | 18,5                  | 1,16    | 16                       | 73 |
| 19. 10. 59          | Müller-Thurgau . . . . . | unbehandelt | 20                       | —                     | —       | —                        | —  |
|                     |                          | Rindite     | 20                       | 21,1                  | 0,90    | 16                       | 80 |
| 19. 10. 59          | Riesling . . . . .       | unbehandelt | 20                       | —                     | —       | —                        | —  |
|                     |                          | Rindite     | 20                       | 19,8                  | 0,78    | 15                       | 75 |

Zu diesem Zweck wurden nun die Knospen von 1jährigen Topfreben, die auf 4 Augen zurückgeschnitten waren, und von 1-Augenstecklingen mittels einer fein ausgezogenen Glaskapillare mit Rindite betropft. Jede Knospe erhielt auf diese Weise im Mittel 4-6 mg Rindite.

Die Ergebnisse mehrerer Versuche sind in den Tabellen I und II zusammengefasst. Stets erwies sich bei allen Sorten Rindite als hochwirksames Agens zur Aufhebung der Knospenruhe. Von den in diesem Gemisch enthaltenen Komponenten war Äthylenchlorhydrin genau so wirksam wie Rindite, während Äthylchlorid<sup>6</sup> eine nur sehr schwache Austriebsförderung besass. Die unbehandelten Kontrollen trieben nur sehr spärlich (Tabelle I) oder auch nach mehrwöchentlicher Versuchsdauer erwartungsgemäss nicht aus. Bei zeitiger Anwendung (Ende September) führten höhere Gaben – mehrmalige tropfenförmige Applikation in Abständen von 12 und 24 h – zu einer leichten, zeitlichen Austriebsverzögerung. Nur vereinzelt wurden hierbei geringfügige Blattschäden beobachtet, die aber mit fortschreitendem Wachstum sehr bald völlig überwunden wurden. Die Wurzelentwicklung der Stecklinge war normal. Unterschiedliche Sortenreaktionen können zum Teil auf die verschiedene Geschlossenheit und Dichte der Knospenschuppen zurückgeführt werden, da sie das Eindringen von Rindite in das Knospengewebe erschweren.

Wirksam erwies sich auch die Behandlung von Stecklingen mit 2 ml Rindite/1 kg Stecklingsfrischgewicht in einem geschlossenen Glasbehälter (Inhalt 2 l) für die Dauer von 24 h und einer Temperatur von 28°C. Eine gleichzeitige Behandlung mit Gibberellinsäure (1 mg auf 1 g Lanolin) erwies sich wieder als austriebshemmend.

Durch Verwendung von Rindite oder Äthylenchlorhydrin besteht somit die Möglichkeit, den Knospenausrieb von Reben auch während der Winterruhe herbeizuführen, was besonders für eine Reihe wissenschaftlicher Untersuchungen<sup>7</sup> von Interesse ist.

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Summary

Rindite and ethylene chlorhydrin have a promotive effect on breaking dormant buds of grape cuttings and of one year old pot-grown plants. The optimal dosis was approximately 6 mg Rindite per bud.

<sup>6</sup> Äthylendichlorid wurde in diesen Versuchen durch Äthylenchlorid ersetzt.

<sup>7</sup> R. J. WEAVER, Nature 183, 1198 (1959).



## Skeletal Lesions Following Ingestion of Fluoridated Water

Clinical and experimental trials have disclosed that administration of fluorides may give rise to lesions of various skeletal and dental tissues. Mainly in the form of fluorapatite<sup>1,2</sup>, the fluorine apparently accumulates in the osseous tissues, and the spinal column is one of the first places where it is found. It has long been known that intake of fluorine in large amounts – either added to drinking water or by inhalation of fluoride-containing dust – induces a characteristic disease entity which is readily diagnosed clinically and resembles spondylosis. In the skeletal system, this condition is manifested as osteosclerosis<sup>3</sup> and in the teeth as so-called mottled enamel. According to a team of U. S. investigators, the production of gross skeletal lesions by fluoridated drinking water requires of a dose exceeding 4 mg of fluorine/l<sup>4</sup>. Judging by animal experiments, however, it seems as though these osseous lesions set in long before they can be diagnosed clinically<sup>2</sup>. Moreover, there is a dearth of evidence regarding the action on the skeleton of prolonged administration of small doses of fluorine.

As a rule, skeletal lesions have been induced in experimental animals by administration of fluorine in large amounts over a short period, typically the very high dosage of 50 mg/kg body weight daily<sup>5</sup>. The most recent findings with respect to the effect upon growing bone of fluorine would seem to be contained in a Japanese report dated 1959<sup>6</sup>, where it is claimed that skeletal growth is inhibited by high doses and promoted by moderate doses of fluorine.

**Experimental.** Male and female albino rats of the same strain were used for the experiments. Two groups originally comprising 10 rats were, from birth, given exclusively distilled water to drink which contained respectively 20 and 40 mg of fluorides/l. They were allowed to drink this water *ad libitum* and were otherwise fed a standardized, fluoride-free rat bread. A group of 10 controls were fed the same bread and plain distilled water. At approximately quarterly intervals one rat from each group was sacrificed and samples for analysis were taken from teeth, mandible, lumbar spine, thigh, and one hind leg. The offspring of the 2<sup>nd</sup> and 3<sup>rd</sup> generation were similarly treated.

The bone specimens were embedded in methyl methacrylate and sawn into pieces convenient for preparing plane-parallel sections of great planeness and smoothness<sup>7</sup>. These sections were placed on a photographic emulsion (Kodak Spectroscopic Plate No. 649) and exposed to X-rays in the wavelength band around 3 Ångström units (the so-called K absorption edge of calcium is situated at 3 Å). The transmittancy of the organic matrix is very high at this wavelength and so is the absorption of calcium. Consequently, the density of the blackened film will be roughly inversely proportional to the calcium content of the specimen. The white areas of the film represent calcium deposits and hence the dark ones correspond to less mineralized portions. The X-rays were generated in a Matchlett Type OEG-50A tube.

With the degree of water fluoridation adopted, no unmistakable gross radiographic lesions were apparent. The microradiograms nevertheless disclosed distinct differences between the groups receiving fluoridated water and the control group in the degree of skeletal mineralization. However, such dissimilarities were apparent only in sections from the spine and not in those from other parts

of the skeleton. Normally osseous tissue includes Haversian systems with varying degrees of mineralization (Fig. 1), as was the case in the controls. The corresponding sites in the rats given fluoridated water were much more uniformly mineralized, and low-mineralized osteons were exceedingly rare or absent altogether in these groups. In other words, X-ray microscopically visible osteosclerosis would seem to have set in (Fig. 2).

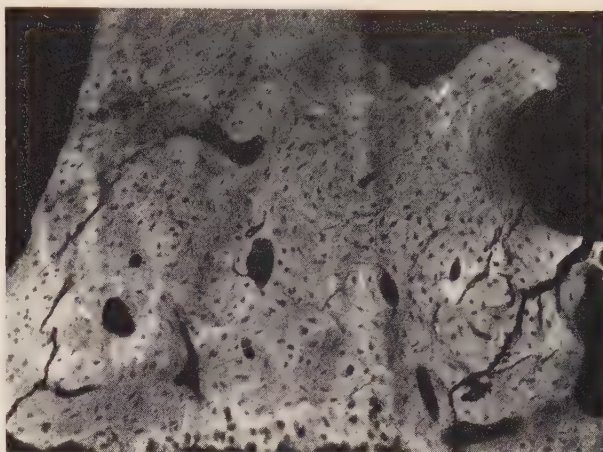


Fig. 1.—Microradiogram showing unequally mineralized Haversian systems in the spine from a control rat

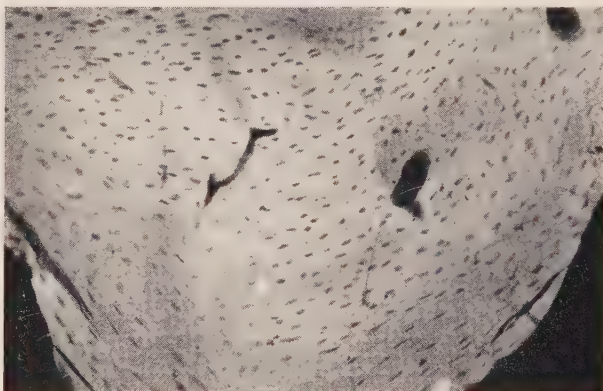


Fig. 2.—Microradiogram presenting distinctly osteosclerotic appearances in the spine from rat on fluoridated water

<sup>1</sup> P. A. BISHOP, Amer. J. Röntgenol. 35, 577 (1936).

<sup>2</sup> W. F. NEUMAN, M. W. NEUMAN, E. R. MAIN, J. O. LEARY, and F. A. SMITH, J. biol. Chem. 187, 655 (1950).

<sup>3</sup> P. F. MÖLLER and S. V. GUDJONSSON, Acta radiol. scand. 13, 268 (1932).

<sup>4</sup> C. A. STEVENSON and A. R. WATSON, Amer. J. Röntgenol. 78, 13 (1957).

<sup>5</sup> E. J. LARGENT, W. MACHLE, and I. F. FERNEAU, J. indust. Hyg. Toxicol. 25, 396 (1943).

<sup>6</sup> K. ABE, Shikoku Acta medica 14, 61 (1959).

<sup>7</sup> O. HALLÉN and H. RÖCKERT, Nature 182, 1255 (1958).

<sup>8</sup> A. ROHOLM, Fluorine Intoxication. A Clinical-Hygienic Study (Lewis & Co., Ltd., London 1937).

<sup>9</sup> W. F. RAMSEYER, C. A. H. SMITH, and C. M. MCCAY, J. Geront. 12, 14 (1957).



In the rats given 20 mg/l, as well as in those given 40 mg/l, these lesions initially appeared at the age of 9 or 12 months. Specimens were obtained from three generations in both groups on fluoridated water. Trials with lower dosages are in progress, and in some of these the total amount of ingested fluorides will be assayed.

The fact that lesions could be demonstrated only in the spine of the rats on fluoridated water is well in line with observations reported by previous investigators. For example, in chronic fluorine intoxication gross lesions have been noted on radiograms of the spine and pelvis<sup>1,3</sup>, sites at which the lesions tend to be incapacitating<sup>8</sup>. It seems not unlikely that, in analogy with other animal experiments<sup>9</sup>, our experiments might disclose involvement of skeletal portions other than the spine when they have been going on for a comparatively long period. Accordingly the initial clinical signs of excessive fluorine administration must be anticipated from the spine. It is too early yet to say whether microscopic lesions (corresponding to those encountered in the present investigation) may eventually give rise to clinical manifestations. Mild lumbar disorders are not susceptible to diagnosis in the rat; and findings in rats cannot be directly translated to human beings unless it has definitely been established that every relevant condition is comparable in the two species.

Incidentally, we would draw attention to the suitability of X-ray microscopy as a tool enabling quantitative changes in chemical composition to be coordinated with morphological appearances.

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Zusammenfassung

Mit Hilfe der Röntgenmikroskopie wurde bei Ratten, die während 9–12 Monaten Wasser mit einem Fluorgehalt von 20–40 mg/l getrunken hatten, Veränderungen in der Mineralisierung der Wirbelsäule nachgewiesen. Die Veränderungen sind mit makroskopischer Röntgenuntersuchung nicht nachweisbar.

Electroshock, Brain Serotonin, and Barbiturate Narcosis

In previous papers<sup>1,2</sup> an increase of brain serotonin has been reported in rats submitted to electroshock. Table I shows the different increases in brain serotonin with different methods of dosage, suggesting that other components of the brain are affected by electroshock.

An investigation using the spectrofluorimetric method has been carried out on different parts of the rat's brain to observe a possible difference in the variation of serotonin content 15 min after electroshock<sup>7</sup>.

Other experiments concern the brain serotonin content at different times after electroshock. The figures obtained are summarized in Table II.

Other kinds of convulsant treatments have been studied. The most important variations (increase of 30% in whole brain,  $p < 0.001$ ) have been observed after metrazol administration (70 mg/kg i. p.), while strychnine (1.5 mg/kg i. p.) and hyperoxia induce only a small rise of brain serotonin (respectively 21 and 19% –  $0.02 > p > 0.001$ ) and amphetamine (15 mg/kg i.p.) or anoxia are practically inactive<sup>7</sup>.

Treatments with barbiturates (phenobarbital 120 mg/kg i.p., pentobarbital 35 mg/kg i.o. or hexobarbital 80 mg/kg i. p.) or diphenylhydantoin (100 mg/kg i.p.) or succinylcholine (1 mg/kg i.p.) are unable to antagonize the increase in brain serotonin induced by electroshock. These data indicate that the variation of brain serotonin observed after electroshock could not be related to the convulsive state<sup>8</sup>. This point has also been confirmed by using electroshocks with different intensity, unable to induce typical convulsions, as reported in Table IV.

<sup>1</sup> S. GARATTINI *et al.*, *Exper.* 13, 330 (1957).  
<sup>2</sup> S. GARATTINI *et al.*, in *Psychotropic Drugs* (ed. S. GARATTINI and V. GHETTI, Elsevier Publ. Co., Amsterdam 1957), p. 428.  
<sup>3</sup> G. E. DALGLIESH *et al.*, *J. Physiol.* 120, 298 (1953).  
<sup>4</sup> J. D. GARVEN, *Brit. J. Pharmacol.* 11, 66 (1956).  
<sup>5</sup> S. UDENFRIEND *et al.* *J. biol. Chem.* 215, 337 (1955).  
<sup>6</sup> D. F. BOGDANSKI *et al.*, *J. Pharmacol. exp. Ther.* 117, 82 (1956).  
<sup>7</sup> S. GARATTINI *et al.*, *Atti Soc. Lomb. Sci. Med. Biol.* 13, 306 (1958).  
<sup>8</sup> M. BISIANI *et al.*, *Atti Soc. Lomb. Sci. Med. Biol.* 13, 345 (1958).

Table I

| Method employed                                                                                      | Brain serotonin (γ/g) |                   | Factor of Increase |
|------------------------------------------------------------------------------------------------------|-----------------------|-------------------|--------------------|
|                                                                                                      | Controls              | Electroshock*     |                    |
| Isolated colon of rat <sup>3</sup> . . . . .                                                         | 0.013 ± 4 (27)        | 0.360 ± 7 (27)    | × 27               |
| Isolated uterus of rat with destruction of norepinephrine according to GARVEN <sup>4</sup> . . . . . | 0.25 (4)              | 0.56 (4)          | × 2.2              |
| Spectrophotometer (275 mμ) <sup>5</sup> . . . . .                                                    | 0.97 ± 0.004 (64)     | 3.02 ± 0.28 (28)  | × 3.1              |
| Spectrophotofluorimeter <sup>6</sup> . . . . .                                                       | 0.47 ± 0.003 (40)     | 0.78 ± 0.021 (36) | × 1.6              |

\* Electroshock was always supramaximal (110 V; 0.2 s) and it was obtained by applying electrodes to the ears. In brackets the number of determinations is reported



Table II

| Part of the brain assayed    | Serotonin content (γ/g) |                   | Increase<br>% | p       |
|------------------------------|-------------------------|-------------------|---------------|---------|
|                              | Controls                | Electroshock      |               |         |
| Whole brain . . . . .        | 0.47 ± 0.003 (40)       | 0.78 ± 0.02 (36)  | +66           | < 0.001 |
| Hemispheres . . . . .        | 0.37 ± 0.012 (40)       | 0.53 ± 0.019 (36) | +43           | < 0.001 |
| Cerebellum* . . . . .        | 0.11 ± 0.009 (40)       | 0.17 ± 0.03 (36)  |               | N. S.   |
| Diencephalic part . . . . .  | 0.81 ± 0.02 (40)        | 1.28 ± 0.06 (36)  | +58           | < 0.001 |
| Mesencephalic part . . . . . | 0.56 ± 0.03 (40)        | 1.08 ± 0.06 (36)  | +93           | < 0.001 |

\* The figures obtained are at the minimum limit of sensitivity of the method  
The different parts of the brain were prepared according to a method previously described<sup>7</sup>

Table III

| Time after<br>electroshock | Serotonin content (γ/g) |       |             |       |                      |       |                       |       | Sleeping time after<br>pentobarbital***<br>(min ± S. E.) |      | *     |
|----------------------------|-------------------------|-------|-------------|-------|----------------------|-------|-----------------------|-------|----------------------------------------------------------|------|-------|
|                            | Whole brain             | *     | Hemispheres | *     | Diencephalic<br>part | *     | Mesencephalic<br>part | *     |                                                          |      |       |
| Controls . .               | 0.47 ± 0.01             | —     | 0.38 ± 0.01 | —     | 0.78 ± 0.02          | —     | 0.59 ± 0.02           | —     | 65 ± 2.7                                                 | (55) | —     |
| 1 min . .                  | 0.54 ± 0.01             | +15   | 0.47 ± 0.02 | −23   | 0.87 ± 0.03          | +10   | 0.71 ± 0.04           | +20   | —                                                        | —    | —     |
| 10 min . .                 | 0.64 ± 0.09             | +36** | 0.55 ± 0.02 | +45** | 1.21 ± 0.06          | +56   | 0.89 ± 0.05           | +51   | 86 ± 5.4                                                 | (20) | +32** |
| 20 min . .                 | 0.69 ± 0.02             | +47** | 0.53 ± 0.01 | +40** | 1.28 ± 0.06          | +61** | 1.08 ± 0.06           | +86** | 116 ± 7.5                                                | (15) | +80** |
| 30 min . .                 | 0.58 ± 0.01             | +24** | 0.51 ± 0.05 | +34   | 1.09 ± 0.08          | +39** | 0.82 ± 0.07           | +39   | 113 ± 6                                                  | (15) | +74** |
| 1 h . . .                  | 0.52 ± 0.01             | +11   | 0.56 ± 0.02 | +42** | 1.10 ± 0.02          | +40** | 0.86 ± 0.01           | +46** | 107 ± 7.0                                                | (15) | +65** |
| 4 h . . .                  | 0.54 ± 0.01             | +15   | 0.48 ± 0.01 | +18   | 1.00 ± 0.05          | +28** | 0.81 ± 0.04           | +36   | 82 ± 7.0                                                 | (10) | +26   |
| 8 h . . .                  | 0.51 ± 0.03             | +8    | 0.52 ± 0.01 | +37   | 0.88 ± 0.03          | +12   | 0.76 ± 0.03           | +29   | —                                                        | —    | —     |
| 12 h . . .                 | 0.50 ± 0.07             | +6    | 0.49 ± 0.02 | +29   | 0.98 ± 0.01          | +25** | 0.72 ± 0.03           | +24   | 73 ± 6.5                                                 | (10) | +22   |
| 24 h . . .                 | 0.45 ± 0.01             | −4    | 0.41 ± 0.01 | +8    | 0.84 ± 0.04          | +7    | 0.65 ± 0.04           | +10   | 50 ± 7.5                                                 | (20) | −23   |
| 26 h . . .                 | 0.49 ± 0.01             | +4    | 0.41 ± 0.01 | +8    | 0.82 ± 0.06          | +3    | 0.66 ± 0.09           | +12   | 85 ± 4.8                                                 | (15) | +31   |
| 48 h . . .                 | 0.55 ± 0.02             | +17** | 0.37 ± 0.01 | −3    | 1.01 ± 0.01          | +29** | 0.73 ± 0.01           | +25   | 91 ± 4.4                                                 | (20) | +40** |
| 60 h . . .                 | 0.57 ± 0.01             | +21** | 0.48 ± 0.01 | +26   | 1.07 ± 0.07          | +37** | 0.81 ± 0.02           | +35   | 100 ± 5.2                                                | (25) | +54** |
| 72 h . . .                 | 0.56 ± 0.01             | +19** | 0.50 ± 0.09 | +32** | 1.11 ± 0.05          | +41** | 0.83 ± 0.02           | +41** | 98 ± 4.0                                                 | (20) | +46** |
| 96 h . . .                 | 0.55 ± 0.01             | +17** | 0.48 ± 0.01 | +26   | 1.12 ± 0.03          | +43** | 0.82 ± 0.01           | +39** | 76 ± 5.6                                                 | (10) | +17** |
| 120 h . . .                | 0.53 ± 0.01             | +13   | 0.47 ± 0.01 | +23   | 0.93 ± 0.03          | +19   | 0.71 ± 0.03           | +22   | 58 ± 10.1                                                | (10) | −11   |
| 144 h . . .                | 0.54 ± 0.01             | +15   | 0.46 ± 0.01 | +21   | 0.92 ± 0.01          | +18   | 0.77 ± 0.03           | +31   | 58 ± 5                                                   | (10) | −11   |

\* Percentual change respect to controls  
\*\* p < 0.001  
\*\*\* Pentobarbital was injected i. p. at the concentration of 25 mg/kg. In brackets the number of animals used is reported  
Each figure represents the average of at least 8 determinations for the whole brain, and the average of 4 determinations for the different encephalic parts

A further aspect of this investigation concerns a detailed analysis of the relationship between change in brain serotonin and duration of barbiturate sleeping-time after electroshock. The results obtained are summarized in the last column of the Table III.

Table IV

| Number<br>of rats | Voltage<br>applied (time<br>= 0.2 s) | Convulsions | Serotonin content<br>(γ/g) mesencephalic part |
|-------------------|--------------------------------------|-------------|-----------------------------------------------|
| 16                | —                                    | —           | 0.59 ± 0.02                                   |
| 8                 | 30                                   | —           | 0.82 ± 0.01*                                  |
| 8                 | 50                                   | —           | 0.86 ± 0.02*                                  |
| 8                 | 70                                   | ±           | 0.87 ± 0.05*                                  |
| 8                 | 90                                   | +           | 0.85 ± 0.02*                                  |
| 8                 | 110                                  | ++          | 0.93 ± 0.03*                                  |
| 8                 | 130                                  | ++          | 0.84 ± 0.03*                                  |
| 8                 | 150                                  | ++          | 0.91 ± 0.03*                                  |

\* p < 0.01

These data, showing a good parallelism between changes in brain serotonin and variations of sensitivity to barbiturate after electroshock, could be related to other investigations reporting an anticonvulsant effect exerted by monoamineoxidase inhibitors<sup>9</sup> and demonstrating an increase in brain serotonin after barbiturate treatment<sup>10,11</sup>.

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Riassunto

Dopo elettroshock si osserva nell'encefalo di ratto un aumento di serotonina, particolarmente evidente nell'area mesencefalica e diencefalica, che si accompagna ad una maggior sensibilità al pentobarbital.

<sup>9</sup> D. J. PROČKOP *et al.*, *Exper.* 15, 145 (1959).  
<sup>10</sup> E. G. ANDERSON *et al.*, *Abstr. Fall Meeting Amer. Soc. Pharmacol., Ann. Arbor (Mich.)*, 1958, p. 1.  
<sup>11</sup> D. D. BONNYCASTLE *et al.*, *Brit. J. Pharmacol.* 12, 228 (1957).



## Relation entre la complexion de l'histamine par les carbures cancérigènes et l'effet solvant

Le véhicule dans lequel une substance cancérigène est administrée entraîne généralement des modifications dans l'activité de la dite substance<sup>1-4</sup>.

Cette «action solvant» complexe, multiple et contradictoire dans l'ensemble des cas apparaît nette et claire pour certains composés lipohydrophiles non ioniques du type «Tween» (Tw)<sup>5</sup> et poly-éthylène-glycol (PEG)<sup>6-9</sup>. RISKÀ et SETALA, en particulier, leur ont consacré une étude approfondie et sont arrivés à grouper des résultats concluants quant à l'existence de l'action solvant<sup>6,7</sup>.

Dans les expériences de RISKÀ et SETALA, le 3,4-benzopyrène (B) et le 9,10-diméthyl-1,2-benzanthracène (DMBA) appliqués localement sont rendus considérablement plus actifs lorsqu'ils sont dissous dans les Tw. Au contraire, la dissolution des corps cancérigènes dans les PEG empêche totalement la cancérogenèse dans des conditions identiques. RISKÀ et SETALA signalent aussi que la concentration du solvant modifie l'action de celui-ci d'une manière inattendue.

L'effet solvant, évident quant à son existence, est demeuré obscur quant à ses causes et à son processus<sup>10,11</sup>.

Etant donné la fixation élective des substances cancérigènes par l'histamine (Hi)<sup>12</sup>, on pouvait se demander s'il n'existait pas un lien entre l'effet solvant et cette réaction.

La recherche actuelle montre que ce lien est réel et, pour établir une telle réalité, elle s'appuie sur les expériences décisives de RISKÀ et SETALA. L'étude concerne également B et DMBA. Les solvants choisis sont les Tw<sub>20</sub>, Tw<sub>80</sub>, PEG<sub>400</sub>, PEG<sub>600</sub>, PEG<sub>1500</sub> qui figurent parmi ceux utilisés dans les expériences de RISKÀ et SETALA et qui, dans les conditions d'observations, donnent des milieux transparents se prêtant à l'examen dans l'ultra-violet. Les produits ont la même origine que ceux des auteurs cités. L'expérience est conduite en milieu aqueux car c'est là que les échanges biologiques se produisent.

Les flacons d'étude sont répartis en 5 séries:

- Série I: substance cancérigène + solution de Hi;
- Série II: substance cancérigène + solvant Tw + eau;
- Série III: substance cancérigène + solvant PEG + eau;
- Série IV: substance cancérigène + solvant Tw + solution de Hi;
- Série V: substance cancérigène + solvant PEG + solution de Hi.

Les taux varient de 0,01 à 0,5% pour la substance cancérigène, de 0,05% à 50% pour le solvant, de 10 à 40% pour Hi. La durée du contact est de 2 jours à 20 jours, la température: 25°C à 40°C. Les mélanges sont examinés dans l'ultraviolet.

L'examen spectral donne les résultats suivants:

- Série I: le complexe histamine-substance n'apparaît tout au plus qu'à l'état de traces faibles.
- Série II: une solubilisation franche de la substance se produit.
- Série III: ne donne pratiquement rien.
- Série IV: une solubilisation de la substance se produit mais plus nette et plus grande que dans la série II. Les valeurs données par la série IV et par la série II présentent un écart d'autant plus considérable que Hi est plus concentré (Figures 1 et 2).
- Série V: la solubilisation de la substance se produit dans certains cas particuliers.

Etudiés aux concentrations où ils ont inhibé la tumorigenèse dans les expériences de RISKÀ (concentration: 25%) et observés dans les mêmes conditions que les Tw, les PEG ne font apparaître ni la substance, ni le complexe histamine-substance. Il y a lieu de signaler cependant que, pris un taux plus élevé (50%) leur comportement est différent: la réaction se produit faiblement. Mais, si l'on ramène ainsi à 25% la concentration maximale du solvant par addition d'eau ou bien d'histamine aqueuse dans le même flacon, la substance précipite et la réaction de l'histamine est interrompue même si le contact se prolonge de 8 jours à 40°C. On rejoint ici encore RISKÀ et SETALA lorsqu'ils prévoient que la concentration du véhicule modifie l'action de celui-ci<sup>7</sup>.

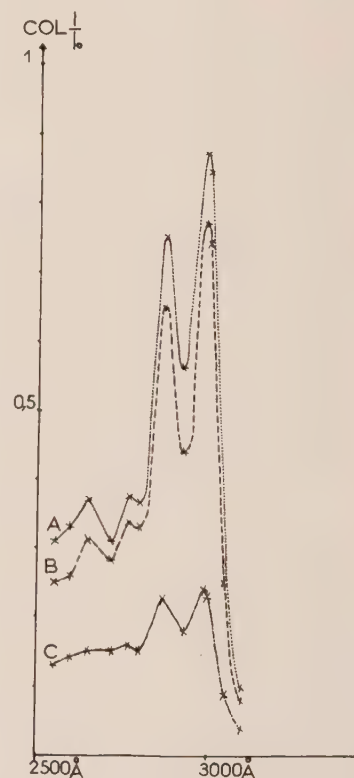


Fig. 1. Densité optique observée sous 0,01 mm d'épaisseur, par rapport au milieu contenant DMBA

Courbe A. DMBA (0,025%), Tw<sub>80</sub> (8%), Hi (2 M) – 19 jours de contact.  
 Courbe B. DMBA (0,025%), Tw<sub>80</sub> (8%), Hi (1 M) – 19 jours de contact.  
 Courbe C. DMBA (0,025%), Tw<sub>80</sub> (8%), H<sub>2</sub>O – 19 jours de contact.

<sup>1</sup> H. WEIL-MALHERBE, *Biochem. J.* **40**, 351, 363 (1946).

<sup>2</sup> F. DICKENS, *Brit. med. Bull.* **4**, 348 (1946-1947).

<sup>3</sup> PH. SHUBIK et J. SICE, *Cancer Res.* **16**, 728 (1956).

<sup>4</sup> A. HADDOW, *Brit. med. Bull.* **14**, 94 (1958).

<sup>5</sup> Les Tween sont des esters d'acide gras et d'anhydride de sorbitol condensés sur une chaîne d'oxyde d'éthylène. Le chiffre désigne l'acide gras ayant servi à l'estérification. Ainsi, Tw<sub>20</sub>: acide laurique; Tw<sub>80</sub>: acide oléique, etc.

<sup>6</sup> K. SETALA, P. HOLSTI et S. LUNDBOM, *Acta Unio intern. contra Cancrum* **13**, 280 (1957).

<sup>7</sup> E. B. RISKÀ, *Acta pathol. microbiol. scand., Suppl.* **114**, 34, 45 (1956).

<sup>8</sup> F. BIELCHOWSKY et D. LINDSAY, *34th Rep. Brit. Emp. Canc. Comp.*, p. 370 (1956).

<sup>9</sup> L. BERENBLUM et N. HARAN, *Canc. Res.* **15**, 510 (1955).

<sup>10</sup> E. RISKÀ, *Acta pathol. microbiol. scand., Suppl.* **115**, 78 (1956).

<sup>11</sup> K. SETALA, *Acta Unio intern. contra Cancrum* **13** (1957).

<sup>12</sup> S. HATEM, *Exper.* **15**, 219 (1959).

Le résultat était établi *in vitro*; il appelait une vérification *in vivo*. Cette vérification a été faite avec le concours de CH. CHAMPY. Nous avons montré que les corps cancérogènes placés à la surface d'un organe riche en nerfs histaminiques font disparaître la réaction microchimique de l'histamine sur une certaine profondeur en 48 h<sup>12</sup>. Nous étudions aujourd'hui l'action comparée d'un Tw et d'un PEG sur cette même réaction.

L'expérience est faite sur la sous-maxillaire de rat comme précédemment en appliquant à droite B mêlé au Tw<sub>80</sub>, à gauche B mêlé au poly-éthylène-glycol<sub>400</sub>, toutes proportions égales. A droite, on observe la disparition des nerfs: B a bien fixé l'histamine. A gauche, les nerfs sont parfaitement colorés, leurs plus fines terminaisons et les plus superficielles apparaissent: le PEG a bien protégé l'histamine contre l'action d'une substance active.

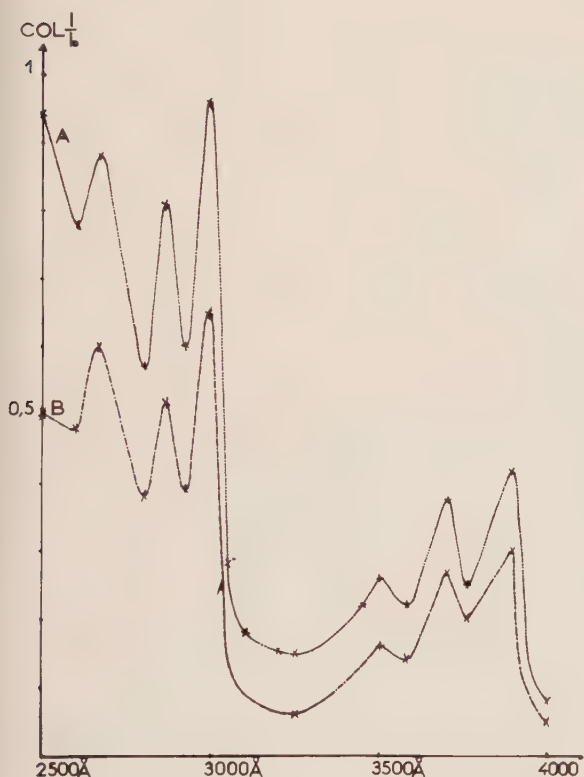


Fig. 2. Densité optique observée sous 0,01 mm d'épaisseur, par rapport au milieu contenant B

Courbe A. B (0,05%), Tw<sub>80</sub> (8%), Hi (1,5M) – 8 jours de contact  
 Courbe B. B (0,05%), Tw<sub>80</sub> (8%), H<sub>2</sub>O – 41 jours de contact

Ce lien entre l'action particulière des solvants sur l'incidence tumorale et la réaction de l'histamine semble confirmer que la captation de l'amine par les substances actives est un élément essentiel de la cancérisation et le solvant se révèle ainsi non seulement un véhicule mais encore un médiateur dont l'action atteint le mécanisme profond de la cancérisation.

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 le 9 novembre 1959.

### Summary

A relationship is observed between the action of certain solvents on cancerogenesis and the affinity of histamine for cancerproducing substances. Tw-type solvents, which increase the tumoral incidence brought about by a chemical substance, intensify the reaction of histamine with this substance; while PEG-type solvents, which retard tumorogenesis, inhibit the reaction.

### Behavioral Effects of 5-Hydroxytryptophan<sup>1</sup>

Many investigators<sup>2-9</sup> have suggested that 5-hydroxytryptamine (serotonin) may have a role in brain function. In studies where animals were given 5-hydroxytryptophan (5-HTP), the brain serotonin levels were elevated<sup>10-13</sup>. 5-HTP is capable crossing the blood-brain barrier and there be converted into serotonin. The animals exhibit central disturbances characterized by tremors, signs of sympathetic stimulation, and behavioral changes<sup>12,13</sup>. Characterization of the latter have been anecdotal. In order to objectively assess the behavioral changes resulting from increased serotonin levels, the behavioral effects of injecting the serotonin precursor, 5-HTP, was studied by measuring the pecking response of the pigeon in a Skinner box<sup>14,15</sup>. In this way, the change in the behavior of the bird, which was being quantitatively as well as continuously measured, could be correlated to the dose of the injected 5-HTP. Ultimately, birds in such an experiment will be sacrificed and changes in their brain serotonin levels compared to changes in their behavioral performance.

A red and white color was alternately present on the key which the bird pecked. When the key was red, the 50<sup>th</sup> peck operated the food magazine (fixed-ratio) giving the hungry bird 3.5 s access to grain. When the key was white (or not colored) the first response after 10 min operated the magazine (fixed-interval). The patterns of responding conformed to the respective reinforcement conditions, and provided a baseline with two repeated patterns, occurring continuously during a 5-hour experimental session<sup>14,15</sup>.

<sup>1</sup> This investigation was supported in part by a research grant MY-3225 from the National Institute of Mental Health, Public Health Service and in part by a grant-in-aid from Eli Lilly and Company.

<sup>2</sup> D. W. WOOLLEY and E. N. SHAW, Proc. Nat. Acad. Sci., Wash. 40, 228 (1954).

<sup>3</sup> J. H. GADDUM, Ciba Colloquium on Hypertension, 75 (1954).

<sup>4</sup> B. B. BRODIE, A. PLETSCHER, and P. A. SHORE, Science 122, 968 (1955).

<sup>5</sup> J. H. GADDUM and M. VOGT, Brit. J. Pharmacol. Chemotherap. 11, 175 (1956).

<sup>6</sup> D. W. WOOLLEY and E. N. SHAW, Ann. N. Y. Acad. Sci. 66, 649 (1957).

<sup>7</sup> D. W. WOOLLEY, Hormones, Brain Function, and Behaviour, (Academic Press, Inc., New York 1957), p. 127.

<sup>8</sup> B. B. BRODIE and P. A. SHORE, Ann. N. Y. Acad. Sci. 66, 631 (1957).

<sup>9</sup> A. S. MARRAZZI, Ann. N. Y. Acad. Sci. 66, 496 (1957).

<sup>10</sup> S. UDENFRIEND, H. WEISSBACH, and D. F. BOGDANSKI, J. biol. Chem. 224, 803 (1957).

<sup>11</sup> K. FRETER, H. WEISSBACH, S. UDENFRIEND, and B. WITKOP, Proc. Soc. exp. Biol. Med., N. Y. 94, 725 (1957).

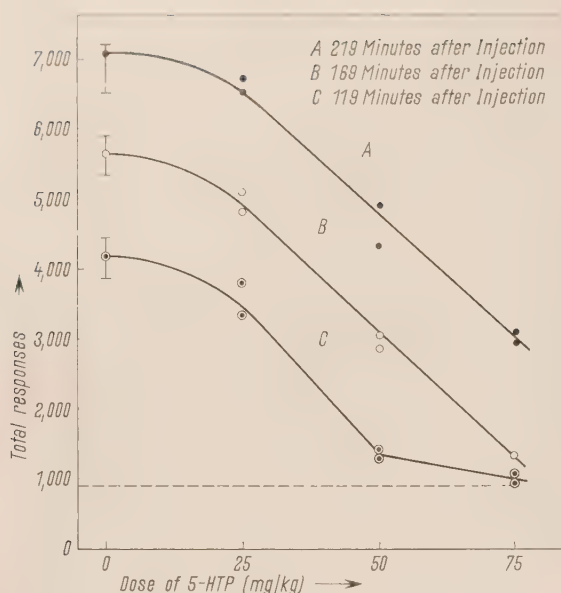
<sup>12</sup> D. F. BOGDANSKI, H. WEISSBACH, and S. UDENFRIEND, J. Pharmacol. exp. Therap. 122, 182 (1958).

<sup>13</sup> E. COSTA and F. RINALDI, Amer. J. Physiol. 194, 214 (1958).

<sup>14</sup> P. B. DEWS, J. Pharmacol. exp. Therap. 113, 9 (1955).

<sup>15</sup> C. B. FERSTER and B. F. SKINNER, Schedules of Reinforcement (Appleton-Century-Crofts, New York 1957).





Relationship of dose of 5-hydroxytryptophan and amount of responding in one pigeon. Each curve presents the number of responses made in the indicated times after injection. Values are adjusted in terms of the pre-injection levels of responding. Each point represents a single dose, except, in the case of the control and saline values where the median and inter-quartile range are given for 6 sessions. Control sessions were taken from the days preceding each 5-HTP injection

Two groups of pigeons were used; one, in which the 5-HTP was administered orally ( $n = 4$ ) and the other, a group in which the compound was injected intramuscularly ( $n = 4$ ). The results of a typical experiment on a single pigeon in the latter group are shown in the Figure, where the abscissa axis represents the dose of 5-HTP administered (mg/kg) and the ordinate axis the total number of responses made by the bird in a specific interval after injection. The control data was calculated by determining the median and quarter percentile values of data obtained on days when saline or no 5-HTP was administered. Single doses of 25, 50, and 75 mg/kg of 5-HTP were injected into the breast muscle over a period of several months with each dose given at least twice. The injections were given after the third 10-min fixed interval or 31½ min after the start of the daily session. The initial period also included three fixed ratios alternating with fixed intervals and each taking approximately 0.5 min. Since our techniques allowed us to measure the performance of the animal just prior to the point of the injection, it was possible to adjust the subsequent performance by correcting for differences in the initial level of responding. Curve A, B, and C give the number of responses made by the pigeon 219, 169, and 119 min after the injections of 5-HTP or saline. If more curves were plotted at decreasing times, the curves would approach the dotted line on 900 responses, the adjusted number of responses at the time of the injection. With increasing doses of 5-HTP, the total number of pecks in the specified intervals decreased.

Following the injection, the birds usually complete the next fixed-ratio. The onset of the effect of the injection occurs approximately 1–5 min later in the fixed-interval. In general, the fixed-ratio components of the multiple performance were not affected at doses which severely disrupted the fixed-interval performances. At highest doses and at peak effects, however, the fixed-ratio performance was also altered. Data on other pigeons in the same group were similar in nature.

When the 5-HTP was administered orally, the same type of relationship was obtained except that higher doses of 5-HTP were required to produce a decrease in pecking. The appearance of the behavioral effect of the 5-HTP injection occurred later than when administered intramuscularly.

Studies are now under way in which serotonin, 5-hydroxytryptophan, plus Marsilid, and serotonin antimetabolites are being administered to pigeons under similar conditions in an attempt to determine if the behavioral effects observed are central, peripheral, or both.

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#### Zusammenfassung

Bestimmung der Verhaltensänderung bei Tauben, durch Injektion von 5-Hydroxytryptophan, der Vorstufe von Serotonin hervorgerufen. Mit steigender Dosis von 5-Hydroxytryptophan wird eine entsprechende Frequenzverminderung des Pick-Verhaltens ausgelöst.

#### Autoradiographic Study of Cartilage Differentiation in Organ Culture

Recent work has shown that radio-sulphate is taken up specifically by cells and tissues known to contain large amounts of sulphomucopolysaccharides<sup>1</sup>. Studies on young embryos have not only demonstrated a high activity in embryonic cartilage, but have suggested a specificity of precartilaginous cells in high incorporation of the isotype<sup>2</sup>. The present note describes the situation in explanted embryonic somites differentiating in the conditions of organ culture.

**Materials and Methods.** Most of the experiments used chick embryos of 4½ days incubation (stage 24–25<sup>3</sup>), but some 11-day mouse embryos were also employed. Clean isolation of the somitic material was assisted by trypsin treatment<sup>4</sup>. The liquid culture medium was composed of horse serum, modified Tyrode<sup>5</sup> and embryo extract, in proportions 2:2:1. Two or three somites were cultured together on pieces of lens paper<sup>6</sup> which was arranged to cover the top of a plastic well holding 1 ml of medium, the whole assembly being placed in a solid watch glass containing sufficient saline to maintain a moist atmosphere. Labelling was done by placing the lens-paper carrying the culture for an appropriate time over a well containing culture medium containing carrier-free sulphate-S<sup>35</sup> at 4 µc/ml. For comparison, similar experiments were made with mesonephric tissue from the same donors. After fixation in methanol, and sectioning at 5 µ, autoradiographs were made by stripping film (exposure 15 days at 4°C), some parts of each ribbon being left without film as controls.

<sup>1</sup> D. D. DZIEWIATKOWSKI, *Int. Rev. Cytol.* 7, 159 (1958).

<sup>2</sup> R. AMPRINO, *Acta anat.* 24, 121 (1955). – U. FRIBERG and N. R. RINGERTZ, *J. Embryol. exper. Morphol.* 4, 313 (1956). – P. M. JOHNSTON and C. L. COMAR, *J. biophys. biochem. Cytol.* 3, 231 (1957).

<sup>3</sup> V. HAMBURGER and H. HAMILTON, *J. Morphol.* 88, 49 (1951).

<sup>4</sup> G. AVERY, M. CHOW, and H. HOLTZER, *J. exper. Zool.* 132, 409 (1956).

<sup>5</sup> M. JONES and S. L. BONTING, *Exper. Cell Res.* 10, 631 (1956).

<sup>6</sup> J. M. CHEN, *Exper. Cell Res.* 7, 518 (1954).

**Results.** The specimens which were labelled with radiosulphate for 16 h gave sufficient density of grains for a qualitative description to be based on a subjective inspection of the autoradiographs. After labelling for 2.5 h, the activity was not high enough to make a definite estimation, although the results were generally similar to those from the specimens with 16 h labelling. No essential difference was noted between the cultures of mouse and chick somites.

In the specimens labelled for 16 h immediately after the isolation of the somites, and then fixed, marked accumulation of the isotope in the chondrogenic blastema was already quite indisputable, although a fairly high activity was found all over the tissue fragment (Fig. 1). In the chondrogenic area the grains were mainly distributed in the cytoplasm of the precartilaginous cells and in the intercellular space, while the activity in the nucleus was very doubtful. It is noteworthy that the cytoplasmic activity was estimated to be relatively higher than intercellular activity. The number of the grains per single precartilage cell was counted as 3.2 times as many as those per single non-chondrogenic cell in the somites. On observation by conventional histological methods, however, the chondrogenic area of the present specimens appeared merely as a compact assembly of undifferentiated precartilaginous cells, no metachromatic substance being detectable after staining with toluidine blue. Thus, the selectivity of the precartilaginous blastema for radiosulphate, which was manifested both in the intact animal<sup>7</sup> and in the dispersed tissue culture<sup>8</sup>, can be well confirmed in the condition of organ culture. The specificity of the precartilage cells has also been demonstrated by an im-

munological approach, in which the positive reaction of these cells to fluorescent labelled antiserum against the differentiated cartilage has been observed.

After another 48 h' cultivation in non-radioactive medium (total culturing time, ca. 64 h), cartilage with metachromatic matrix was developed; and the radiosulphate taken up in the initial labelling was well maintained in this tissue (Fig. 2). On the other hand, a slight loss of activity was noted in the non-chondral tissues. As a result, the localization of radiosulphate in the cartilage appears to be very precise. Observation under high magnification revealed that the activity of the intercellular material was very much intensified in comparison with the previous stage, whereas the intracellular activity became lower. This must indicate a shift of the isotope from the interior of the precartilage cells into the intercellular matrix of the newly differentiated cartilage.

In some cases, cultivation in non-radioactive medium was prolonged up to 96 h after labelling. The autoradiography of these cases shows the preservation of radiosulphate in the cartilaginous matrix, only a very small number of grains being found on the cells. But, as a general impression, the density of grains per unit area of the matrix appeared less than that in earlier stage. The swelling of the intercellular matrix caused by the deposition

<sup>7</sup> R. AMPRINO, *Acta anat.* 24, 121 (1955). – P. M. JOHNSTON and C. L. COMAR, *J. biophys. biochem. Cytol.* 3, 231 (1957).

<sup>8</sup> R. E. MANCINI, L. NUEZ, and E. S. LUSTING, *J. Histochem. Cytochem.* 4, 444 (1956).

<sup>9</sup> T. S. OKADA and R. M. CLAYTON, in preparation.

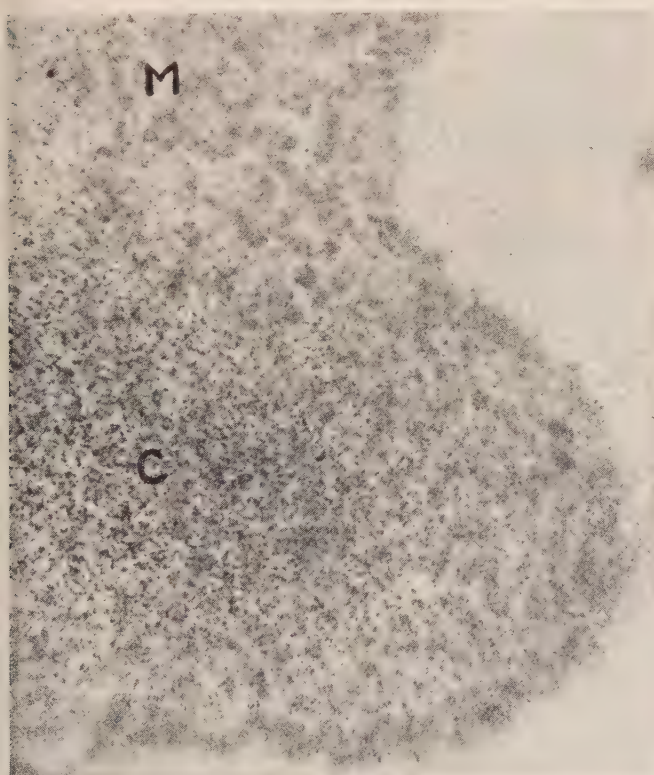


Fig. 1. – Autoradiograph of the culture fixed immediately after labelling in the initial stage of culturing. High uptake of radiosulphate is seen in the chondrogenic blastema, the grains being mainly distributed over intracellular sites (Haematoxylin and diluted eosin, chick)

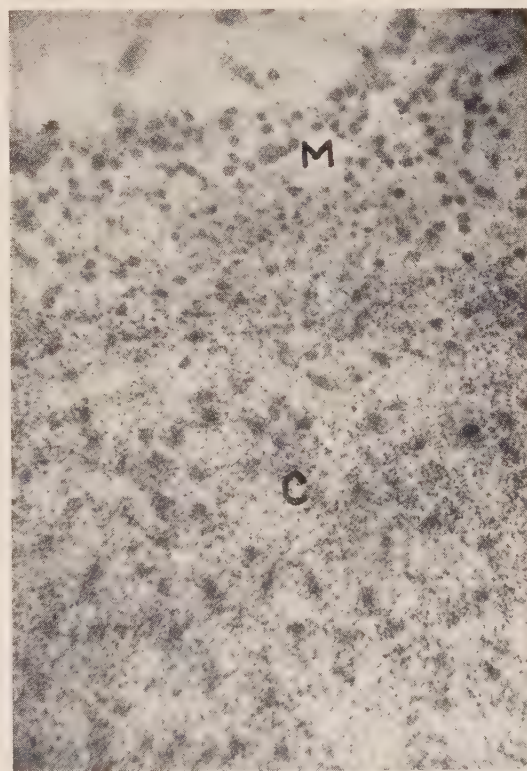


Fig. 2. – Autoradiograph of the culture fixed 48 h, after labelling in the initial stage. Note that the intercellular activity is higher than in the previous stage shown in Figure 1 (Haematoxylin and diluted eosin, chick)

C: Precartilaginous (in Fig. 1) or cartilaginous area (in Fig. 2). M: Myogenic area



of newly synthesized ground substance would bring about the dispersal of the grains per unit area; thus the actual decrease in the total activity in the cartilage may not be as great as suggested by the grain density.

Mesonephric tissue also picked up some radiosulphate under organ culture conditions, and the activity was still preserved after 2 days' culturing in non-radioactive medium, by which time typical mesonephric tubules were formed. The activity of this tissue, however, was less than that of even the non-chondral components of somites, and far below that of the chondrogenic tissue. This fact demonstrates that the specificity of the uptake of radiosulphate by different kinds of tissue is well maintained in isolated tissue fragments.

**Discussion.** The uptake of radiosulphate by cells and subsequent movement into matrix have been previously shown in the differentiated cartilage of the adult mouse<sup>10</sup> and of the late chick embryo<sup>11</sup>. The present results indicate that a similar shift of tracer takes place in the initial differentiation of precartilage into cartilage with metachromatic matrix.

No metachromatic substance is detectable in the precartilaginous tissue, and it therefore seems likely that the substance into which the isotope is incorporated is a precursor of the sulpho-mucopolysaccharides of the future matrix. The existence of such a precursor has been suggested from the results of PAS staining<sup>12</sup>, while it has also been claimed that chondroitin sulphate does not appear until after cartilage becomes histologically differentiated<sup>13</sup>.

**Acknowledgments.** The author wishes to thank Prof. C. H. WADDINGTON for suggesting this problem and for his encouragement; he is also indebted to Dr. J. L. SURLIN for introducing him to the technique of autoradiography. The author has been the holder of a Macauley Fellowship from Edinburgh University, which has made his research in the U. K. possible.

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*Institute of Animal Genetics, Edinburgh, August 17, 1959.* \* On leave of absence from the Zoological Institute, Faculty of Science, University of Kyoto (Japan).

### Zusammenfassung

Aufnahme und Verhalten von <sup>35</sup>S-Sulfat wurde in einer Organkultur sich differenzierender Somiten von Hühner- und Mäuse-Embryonen untersucht. Die Vorknorpelzellen zeigten den grössten Isotopeneinbau im Vergleich zu andern mesodermalen Zellen. Nach Vollendung der Knorpeldifferenzierung fand sich das von den Vorknorpelzellen aufgenommene Isotop in der interzellulären Matrix.

<sup>10</sup> S. R. PELC and A. GLUCKSMAN, *Exper. Cell Res.* **8**, 336 (1955).

<sup>11</sup> H. B. FELL, E. MELLANBY, and S. R. PELC, *J. Physiol.* **134**, 179 (1956).

<sup>12</sup> A. MOSCONA and H. MOSCONA, *J. Anat.* **86**, 287 (1952).

<sup>13</sup> H. HOLTZER, Oral communication at UNESCO-Symposium (Edinburgh 1957): *Biological Organisation* (Ed. C. H. WADDINGTON, Pergamon Press, London), in press.

### PRO LABORATORIO

#### An Adjustable Synchronized Electron Flash for Phase Contrast Micrography

The growing use of phase contrast microscopy for the study of brain tissue made a difficulty which was felt in the photographic recording of the pictures obtained. In this form of microscopy, the preparations studied are often not fixed and usually show intense Brownian movement, which may make an extremely short exposure time indispensable. However, this demand is very difficult to meet, since with the same source of light, the pictures have a lower light intensity than those of common microscopy with diaphenous light. Phase contrast microscopy therefore often requires flash exposure with extra strong light intensity. The commercially available flash apparatus for micrography are not suitable for this purpose, because the exposure time cannot be regulated

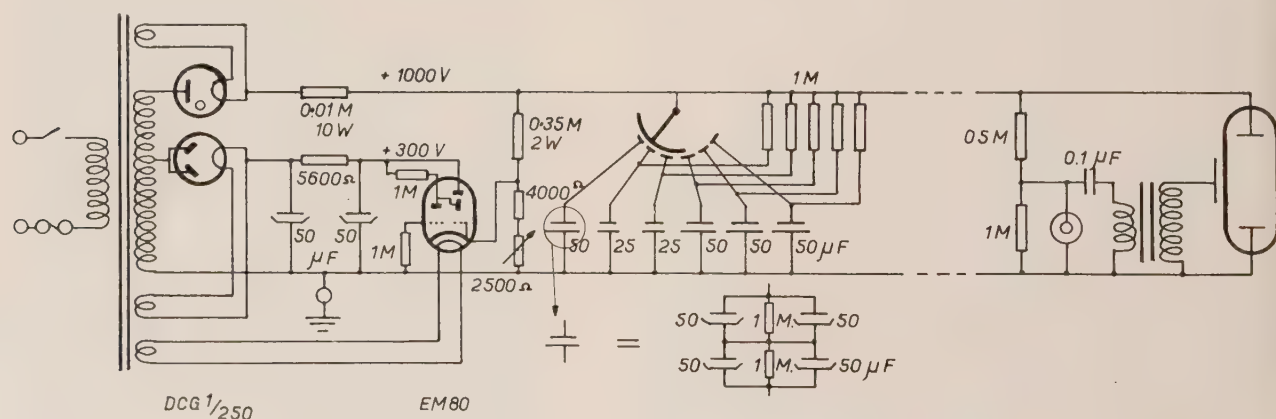


Fig. 1.—Wiring scheme of an electron flash for phase contrast microscopy

A double rectifier delivers two voltages one of + 1000 V and another of + 300 V with regard to a common negative pole. Between the output of + 1000 V and earth, a set of condensers is connected with a total capacity of 50  $\mu$ F. With the aid of a switch, one to six condensers of 50  $\mu$ F can be added in parallel. To prevent sparks, which occur when unloaded condensers are added to the original set by means of the switch, the positive pole of each condenser is connected via a resistor of 1 MegOhm with the wire of the + 1000 V.

In this way all condensers are charged to + 1000 V, except very shortly after the discharge.

Between earth and the sparking plug of the electron flash bulb, the secondary coil of a high voltage transformer is connected. The primary coil of this transformer is connected in series with a condenser of 0.1  $\mu$ F and in parallel with a resistor of 1 MegOhm, which is part of a resistor bridge of 1.5 MegOhm between 0 and + 1000 V. One of the poles of the condenser has now in this way a potential of  $\frac{2}{3} \times 1000$  V. The resistor of 1 MegOhm can be short-circuited through a switch, at which moment the potential of this condenser drops suddenly from + 1000 V to 0 V. This discharge causes sufficient potential to ignite the flash bulb. In this circuit, the discharge of the condensers causes an amount of light which is approximately proportional to the total capacity of the condensers.

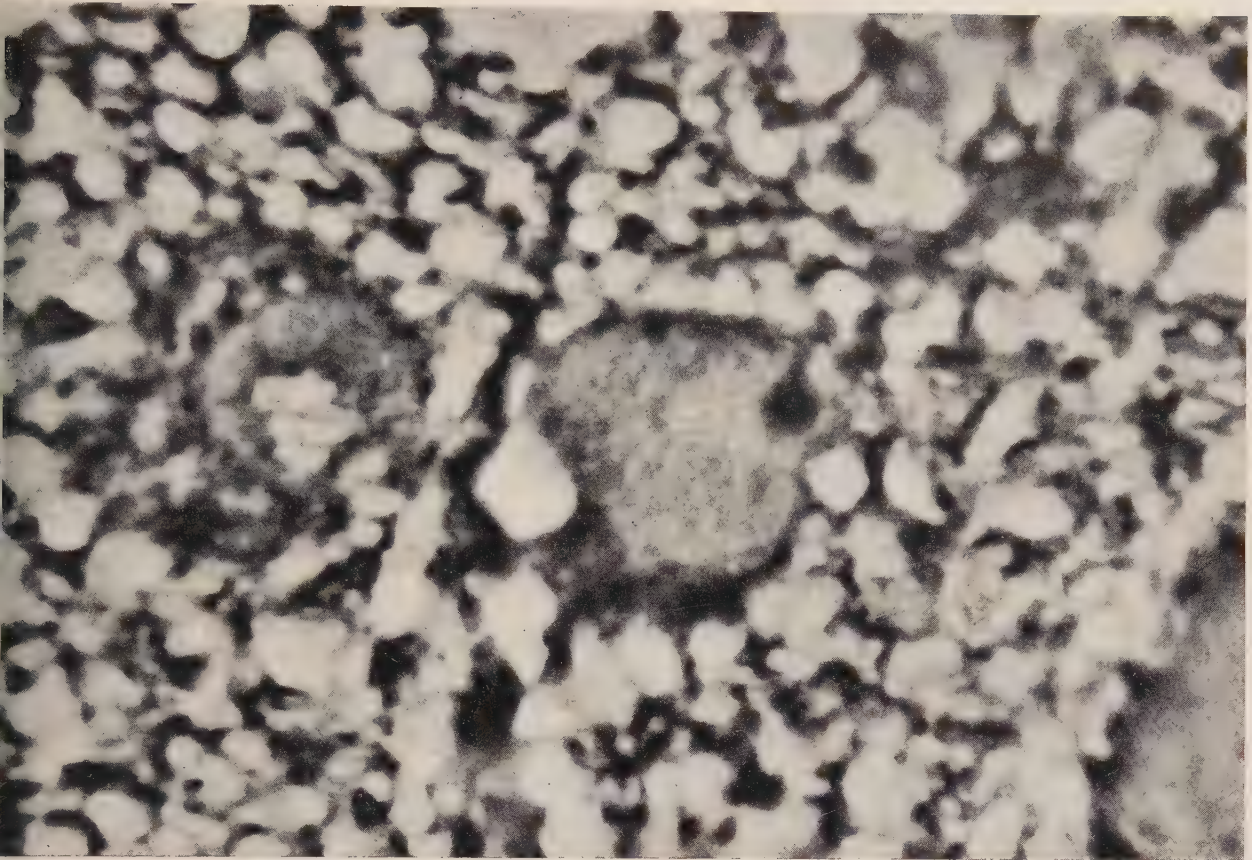


Fig. 2.—Micrograph of freshly prepared nervous tissue of the cerebral cortex of the rabbit taken with the described apparatus and a Zeiss phase contrast microscope. Magnification 2000  $\times$

precisely enough (viz. only by changing filters). A flash apparatus was constructed which lacked these and other failures.

Figure 1 shows a wiring scheme of the electron flash. The lamp used for this apparatus is the electron flash tube of the Phys. Techn. Werkstätten Prof. Dr. Ing. Heimann in Wiesbaden (Germany). This lamp can produce more than 7500 lm sec per flash. The lamp is mounted directly under the condensor lens of the microscope. The mirror of the microscope is replaced by a glass slide, which is pervious to the flash light and reflects most of the light from the microscopic lamp in the usual way.

The light intensity is regulated by a series of condensers, which are connected parallel to the flash tube and having values between 1 and 5 times 50  $\mu$ F. One or several groups of condensers could be discharged, which gave the desired amount of light for the negative of the film. A switch was constructed, which indicated so-called 'flash-factors'.

The Table shows the flash-factors (= position of switch) and the objective-lenses of the microscope. The flashfactors facilitate obtaining negatives with the same density.

Since the ratios of the amount of light for phase contrast lenses were not precisely known, a series of measurements were carried out with a densitometer placed in the tube of the microscope. With the Zeiss phase contrast lenses used, the values measured were approximately inversely proportional to the 2/5 power of the magnification of the objective-lenses. The amount of light necessary to produce the same degree of blackening of the negatives

shows, for the objective lenses 10  $\times$ , 40  $\times$ , 100  $\times$ , a ratio of approximately 2:4:5, which led to the choice of the above-mentioned flash-factors. Figure 2 shows a micrograph of freshly prepared nervous tissue of the cerebral cortex of the rabbit taken with the apparatus described (magnification 2000  $\times$ ).

| Flash-factor<br>(= position of switch) | Objective-lens                            |
|----------------------------------------|-------------------------------------------|
| 1                                      | 10 $\times$                               |
| 1.5                                    | 40 $\times$                               |
| 2                                      | 10 $\times$ phase-contrast                |
| 3                                      | 100 $\times$ oil immersion                |
| 4                                      | 40 $\times$ phase contrast                |
| 5                                      | 100 $\times$ oil immersion phase contrast |

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with the technical assistance of H. OVERDIJK

*Central Institute for Brain Research, University of Amsterdam (Holland), June 4, 1959.*

#### Résumé

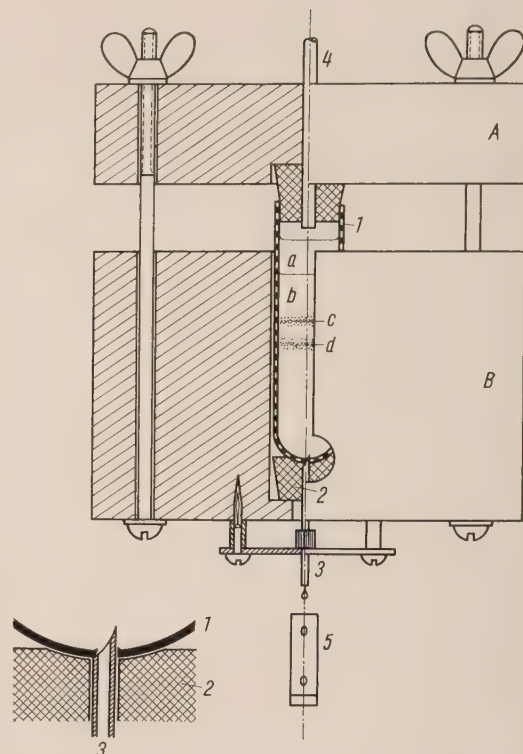
Description d'un appareil à éclair électronique réglable pour la microscopie au contraste de phase. Le circuit présenté permet de régler électroniquement la quantité de lumière par «flash»; ce résultat est obtenu par une diminution ou une augmentation du nombre de condensateurs de 50  $\mu$ F, dans le circuit.



## PRO LABORATORIO

### Sampling of Virus Particles and Macromolecules Sedimented in an Equilibrium Density Gradient<sup>1</sup>

Separation of large molecules on the basis of differences in density has been accomplished by high-speed centrifugation in CsCl equilibrium density gradients<sup>2</sup>. This procedure, originally developed for analytical purposes, was subsequently applied to preparative fractionation of whole viable phage particles<sup>3</sup> and isolated DNA preparations. For the latter purpose, model L of the refrigerated Spinco centrifuge, equipped with the SW-39L swinging-bucket rotor and temperature sensing device PS003, was employed. The position of the phage or DNA layers (*c, d*), formed at their neutral buoyancy levels, was determined by punching a small hole in the bottom of the siliconized centrifuge tube and collecting the effluent droplets individually in a large series of small test tubes pending assay of their content. Neither this fraction collecting operation nor others reviewed by *deDUVE et al.*<sup>4</sup> were found to be entirely satisfactory until the simple instrument depicted in the Figure was devised. The operation, as shown in the Figure, involves collecting the effluent samples through a needle, which penetrates the centrifuge tube from below, in such a manner that (1) stratification of the layers is not disturbed; (2) the tip of the needle barely penetrates the tube, avoiding formation of a 'dead' volume of liquid at the bottom; and (3) a seal is formed around the needle at the point of its introduction. The latter requirement was satisfied by compressing the tube between two blocks of wood or metal (*A* and *B*), which step, while forming a tight seal between the bottom of the centrifuge tube *1* and the soft-rubber stopper *2*, caused the emergence of the tip of the needle *3* from the stopper *2* into the centrifuge tube. The block *B* was so designed that the whole piercing operation could be carefully observed. The rate of drip depended on the air pressure in the tube *1*, which in turn was regulated by manipulating the difference between the water levels in the two vessels connected to the tubing *4*. For most DNA centrifugations, Lusteroid or polypropylene tubes *1* (5 ml; Spinco No. 5050 or 3682), containing 3 ml of CsCl<sup>5</sup> solution *b* overlaid with 1.5 ml of paraffin oil *a* to prevent collapse of the tube, were used. With the proper adaptor (Spinco No. 5527), 0.8-ml tubes (Spinco No. 5528) can be employed. The volume of each drop, using a silicon-treated needle (N. Y. Board of Health needle 19 G, No. 496 NR, Becton, Dickinson & Co., Rutherford, N. J.), was approximately 0.01 ml. The drops were collected in 40 to 120 vials *5* (9 × 30 mm, 1/4-dram shell vials) which were arranged serially in a long supporting block sliding on a track under the needle *3*. The vials were sealed at once with a strip of adhesive tape. For ideal separation of the fractions centrifugation and all operations were performed at 0°C, using ice-water-cooled block *B*, and the outflow rate was kept below 60 drops/min. Higher flow rates caused dispersion of the distribution peaks. With this arrangement, sampling and analysis of the effluent can be either discontinuous or continuous. Information may be obtained concerning the CsCl density gradient (refractive index measurements) and about the distribution of several high molecular weight materials, including viable phage particles<sup>3</sup> (plaque assay) and DNA (ultraviolet light absorption and/or radioactivity determination).



Fraction collector for determining the distribution of macromolecular substances in the equilibrium density gradient established by high-speed centrifugation

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New Brunswick (N. J.), October 12, 1959.*

#### Résumé

La distribution de composés macromoléculaires, stratifiés dans un gradient de densité de CsCl établi par une ultracentrifugation prolongée, est déterminée quantitativement par le recoupage du contenu du tube. Une courte aiguille hypodermique est tout simplement insérée dans le fond du tube. Le contenu du tube s'épanche goutte à goutte. Les fractions de recoupage peuvent être ensuite analysées une à une.

<sup>1</sup> This research was supported in part by the National Science Foundation.

<sup>2</sup> M. MESELSOHN, F. W. STAHL, and J. VINOGRAD, *Proc. nat. Acad. Sci.*, Wash. 43, 581 (1957).

<sup>3</sup> A. W. KOZINSKI and W. SZYBALSKI, *Virology* 9, 260 (1959).

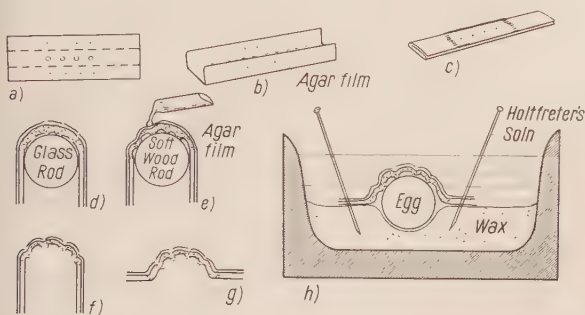
<sup>4</sup> C. deDUVE, J. BERTHET, and H. BEAUFAY, *Progr. Biophys.* 9, 325 (1959).

<sup>5</sup> For DNA sedimentation, a saturated solution of CsCl (0–4°C) in water or in 0.01 *M* *tris* (hydroxymethyl) aminomethane buffer (pH 8) was prepared, and subsequently slightly diluted with water, to obtain a refractive index  $n_D = 1.4120$ . A 2.5 ml quantity of this solution was mixed with 0.5 ml DNA dissolved in 1 *M* NaCl. After 72 h of centrifugation at 35000 r.p.m., the density gradient extended from 1.65 to 1.82 g/cm<sup>3</sup>. The CsCl was purified by repeated recrystallization from boiling water after filtration of a hot solution through activated carbon, until the O. D. at 260 mμ became less than 0.05 as measured against a water blank.

## PRO EXPERIMENTIS

### A Modified Vital Staining Technique for Amphibian Eggs

During a stay at the Hubrecht Laboratory, International Embryological Institute, Utrecht (Holland), the need arose for a technique facilitating the application of regular rows of vital stain marks of different size and spacing on amphibian eggs. The techniques used up till now are best suited for the application of single marks. The number of marks that can be held in position simultaneously is limited in these techniques. HASSA (Cf. NIEUWKOOP c. s. 1955)<sup>1</sup> used a thin collodion film with holes, which was fixed over the egg. By keeping a stained agar block on top of the film, the dye was allowed to pass through the holes in the film, resulting in a row of marks. The technique described here makes use of a strip of stained agar film which is made to protrude through holes in a strip of tin foil.



Various stages in the preparation of tin foil strip to hold stained agar film for vital staining of amphibian eggs.

A very thin agar film is made by pouring melted 3% agar on a clean, polished stainless steel plate. When dry, the film comes off automatically. It is then immersed for 24 h in a 1% solution of Nileblue sulphate, and dried again on the steel plate.

A strip of tin foil of about 15 mm long and 3–4 mm broad is cut out and divided longitudinally into three equal zones by lines (Figure a). Under a dissecting microscope provided with an ocular micrometer, a row of minute holes of proper spacing is made in the middle zone. The edges of the holes are smoothed with a glass rod with a ballend. In the lateral zones, at a level with the holes, small punctures are made, which will later serve as markers. The strip is now folded as shown in Figure b, sterilized in 70% alcohol (about 10 min), rinsed in sterile water, and placed with some water in the groove on a clean, sterile glass slide. A strip of stained agar film of the required size is cut out and placed in the groove. The sides of the tin foil strip are carefully folded one over the other and slightly pressed under another clean glass slide (Fig. c).

The tin foil strip with the agar strip inside is now curved smoothly around a glass rod of the same diameter as the egg (Fig. d). It is then transferred to a rod of soft wood of equal diameter, and, with a fine glass ball, depressions are made in the tin foil over the positions of the holes, indi-

cated by the punctures. Hereby the agar is made to protrude through the holes, and at the same time the parts between the holes become concave (Fig. e and f). Finally the strip is immersed in sterile water, so as to make the agar swell and protrude further.

The staining of the naked egg is illustrated by Figure h. The strip should be treated with gentle pressure only, and the surface of the tin foil should not make contact with the egg surface, since the latter tends to stick to the tin foil. The marks usually attain the required intensity in maximally 30 to 45 min.

With the necessary modifications, this technique may also be applied to the avian, and probably to the reptilian egg.

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Department of Zoology, M. S. University of Baroda (India), July 13, 1959.

#### Zusammenfassung

Es wird eine Methode beschrieben, die es ermöglicht, mit Hilfe eines gefärbten Agarfilms, der durch Löcher in Stanniolpapier gepresst wird, am Amphibien-Ei regelmässige Reihen von Vitalfarbmarken zu setzen.

<sup>1</sup> P. D. NIEUWKOOP *et al.*, Origin and establishment of organization patterns in embryonic fields during early development in amphibians and birds, in particular in the nervous system and its substrate, I, Proc. Kon. Nederl. Akad. Wetensch. Amsterdam [C] 58, 2, 219 (1955).

## PRO EXPERIMENTIS

### Zur Wahl der organischen Lösungsmittel bei der Gewinnung von Chloroplasten in nichtwässrigem Milieu

Die präparative Isolierung morphologischer Zellen- und Gewebebestandteile kann in wässrigem und in nichtwässrigem Milieu (Behrens-Methode) vorgenommen werden. Beide Methoden haben Vor- und Nachteile. Während beim Arbeiten in wässrigem Milieu Fermente, Proteine und andere wasserlösliche Substanzen verloren gehen, wird in nichtwässrigem Milieu den morphologischen Zellbestandteilen ein Teil der Lipide entzogen.

Im Zusammenhang mit den kürzlich beschriebenen Arbeitsgängen zur Gewinnung von Chloroplasten in nichtwässrigem Milieu<sup>1–4</sup> schien es uns von Bedeutung, den Lipidverlust zu ermitteln, welchen gefriergetrocknetes Blattpulver bei Suspension in verschiedenen organischen Solventen erleidet, um die organischen Lösungsmittel mit dem geringsten Lösungsvermögen für Lipide bei der Gewinnung von Chloroplasten heranzuziehen.

<sup>1</sup> M. BEHRENS und R. THALACKER, Naturwissenschaften 44, 621 (1957).

<sup>2</sup> U. HEBER, Ber. dtsch. bot. Ges. 70, 371 (1957).

<sup>3</sup> C. R. STOCKING, Plant Physiol. 34, 56 (1959).

<sup>4</sup> R. THALACKER und M. BEHRENS, Z. Naturf. 14b, 443 (1959).



Als Untersuchungsobjekte wählten wir *Elodea canadensis*, Spinat- und Tabakblätter. Ausser den seither für die Gewinnung von morphologischen Zellbestandteilen aus tierischen und pflanzlichen Zellen benutzten organischen Lösungsmitteln, wie Äther, Chloroform, Benzol, Tetrachlorkohlenstoff, Petroläther, Cyclohexan und Hexan<sup>5-13</sup>, wurden noch einige andere Solventien mit in die Untersuchung einbezogen (Tabelle I).

Tabelle I

|                            | <i>Elodea canadensis</i> * | Spinat** | Tabak*** |
|----------------------------|----------------------------|----------|----------|
| Pentan . . . . .           | 11,4                       | 19,4     | 21,4     |
| Hexan . . . . .            | 11,7                       | 20,2     | 21,4     |
| Heptan . . . . .           | 11,9                       | 20,2     | 20,1     |
| Petroläther (bis 40°C) . . | 10,8                       | 20,2     | 21,4     |
| Petroläther (40-60°C) . .  | 11,9                       | 24,5     | 21,4     |
| Petroläther (60-80°C) . .  | 11,9                       | 23,0     | 20,1     |
| Cyklohexan . . . . .       | 17,8                       | 23,4     | 38,6     |
| Äther . . . . .            | 32,7                       | 37,1     | 45,5     |
| Benzol . . . . .           | 26,6                       | 41,3     | 36,3     |
| Toluol . . . . .           | 30,2                       | 30,9     | 39,9     |
| Xylol . . . . .            | 29,2                       | 34,0     | 39,3     |
| Chloroform                 |                            |          |          |
| (1% Äthanol enthaltend)    | 55,6                       | 57,1     | 67,0     |
| Tetrachlorkohlenstoff . .  | 19,9                       | 38,5     | 31,9     |

\* Junge Sprosse.

\*\* Handelsware; die Mittelrippen wurden vor der Gefriertrocknung entfernt.

\*\*\* Bei dem untersuchten Tabak handelte es sich um die Sorte Havanna IIc. Es wurden junge Blätter von in Blüte stehenden Pflanzen benutzt. Mittelrippen und stark entwickelte Seitenrippen wurden vor der Gefriertrocknung entfernt

Lipidverlust (% Gesamtlipide) durch organische Lösungsmittel. 2 g gefriergetrocknete und pulverisierte Blätter bzw. Sprosse (*Elodea*) wurden mit 100 ml Lösungsmittel 1 h bei Zimmertemperatur geschüttelt. Nach Filtration wurde eine abgemessene Menge des Filtrates im Vakuum zur Trockne verdampft, der Rückstand im Vakuumexsikkator über konz. Schwefelsäure getrocknet und als Lipide gewogen. Bestimmung der Gesamtlipide: 0,5 g gefriergetrocknetes Blatt- bzw. Sprosspulver (*Elodea*) wurden dreimal 30 min mit je 15 ml Methanol-Chloroform-Gemisch (3:1) am Rückflusskühler gekocht. Die filtrierten Extrakte wurden vereinigt und im Vakuum zur Trockne verdampft. Der Rückstand wurde mit kleinen Portionen (insgesamt 20 ml) einer siedenden Petroläther(40-60°C)-Chloroform-Mischung (1:1) extrahiert. Der Extrakt wurde durch Zentrifugieren vom Ungelösten getrennt und im Vakuum bis zur Trockne eingengt. Der Rückstand wurde dann bis zur Gewichtskonstanz im Vakuum über konz. Schwefelsäure getrocknet

Wie aus der Tabelle hervorgeht, ist die Höhe des Lipidverlustes nicht nur von der Art des Lösungsmittels, sondern auch von dem Untersuchungsmaterial abhängig. So werden der von uns als Ausgangsmaterial für die Chloroplastengewinnung gewählten *Elodea canadensis* weniger Lipide entzogen als den Spinat- und Tabakblättern. Von den Solventien mit einem spezifischen Gewicht unter 1,0 lösen die aliphatischen Kohlenwasserstoffe (unter denen keine grösseren Differenzen bestehen) wenig Lipide, während von den beiden untersuchten Lösungsmitteln mit grösserem spezifischen Gewicht Tetrachlorkohlenstoff ein geringeres Lösungsvermögen als Chloroform besitzt. Da für die Isolierung von Chloroplasten in der Regel

Flüssigkeiten mit einem spezifischen Gewicht zwischen 1,25 und 1,40 benötigt werden, sind somit zur Herstellung des genannten spezifischen Gewichtsbereiches am günstigsten die Kombinationen Petroläther (Siedeber. bis 80°C), Pentan, Hexan oder Heptan mit Tetrachlorkohlenstoff; andere Kombinationen, insbesondere die von Äther mit Chloroform, sind weniger geeignet (Tab. II).

Tabelle II

| Spezifisches Gewicht | Äther-Chloroform | Benzol-Tetrachlorkohlenstoff | Petroläther-(60-80°C)-Tetrachlorkohlenstoff |
|----------------------|------------------|------------------------------|---------------------------------------------|
| 1,25                 | 41,3             | 22,3                         | 19,3                                        |
| 1,30                 | 41,7             | 22,0                         | 19,4                                        |
| 1,35                 | 43,3             | 21,8                         | 19,4                                        |
| 1,40                 | 44,3             | 20,8                         | 20,0                                        |

*Elodea canadensis*. Lipidverlust (% Gesamtlipide) durch verschiedene organische Lösungsmittelgemische mit einem spezifischen Gewicht zwischen 1,25 und 1,40. 2 g gefriergetrocknete und pulverisierte Sprosse wurden mit 100 ml Lösungsmittelgemisch 1 h bei Zimmertemperatur geschüttelt. Nach Filtration wurde eine abgemessene Menge des Filtrates im Vakuum zur Trockne verdampft und der im Vakuum über konzentrierte Schwefelsäure getrocknete Rückstand als Lipide gewogen. Bestimmung der Gesamtlipide siehe Tabelle I

Bei dem von uns beschriebenen Verfahren zur Gewinnung von Chloroplasten aus Blättern von *Elodea canadensis*<sup>4</sup> (Zerkleinern der gefriergetrockneten Sprosse und Anreichern der Chloroplasten in Petroläther (Siedeber. 60-80°C), Reinigung der Chloroplasten in einem spez. Gewichtsgradienten unter Verwendung von Petroläther-Tetrachlorkohlenstoff-Mischungen) wird dem Sprosspulver folgende Menge an Lipiden bzw. Lipidbestandteilen entzogen (Tab. III):

Tabelle III

% Gesamt-

| Lipide | Lipid-P | Lipid-N | Chlorophyll |
|--------|---------|---------|-------------|
| 21,2   | 9,8     | 11,1    | 1,9         |

Verlust an Lipiden und Lipidbestandteilen bei der Gewinnung von Chloroplasten aus Blättern von *Elodea canadensis*. N-Bestimmung nach KJELDAHL, Aufschluss der Substanz mit konzentrierter Schwefelsäure und Kaliumpermanganat nach BEET<sup>14</sup>. P-Bestimmung nach Fiske und Subbarow in der Modifikation von LOHMANN und JENDRASSIK<sup>15</sup>. Chlorophyllbestimmung nach ARNON<sup>16</sup>

<sup>5</sup> M. BEHRENS, Hoppe-Seylers Z. 209, 59 (1932).

<sup>6</sup> M. BEHRENS, Hoppe-Seylers Z. 232, 263 (1935).

<sup>7</sup> R. FEULGEN, M. BEHRENS und S. MAHDIHASSAN, Hoppe-Seylers Z. 246, 203 (1937).

<sup>8</sup> M. BEHRENS, Hoppe-Seylers Z. 253, 185 (1938).

<sup>9</sup> A. L. DOUNCE, G. H. TISHKOFF, S. R. BARNETT und R. M. FREER, J. gen. Physiol. 33, 629 (1950).

<sup>10</sup> H. STERN und A. E. MIRSKY, J. gen. Physiol. 36, 181 (1952).

<sup>11</sup> V. ALLFREY, H. STERN, A. E. MIRSKY und H. SAETREN, J. gen. Physiol. 35, 529 (1952).

<sup>12</sup> H. STERN und A. E. MIRSKY, J. gen. Physiol. 37, 177 (1954).

<sup>13</sup> E. R. M. KAY, R. M. S. SMELLIE, G. E. HUMPHREY und J. N. DAVIDSON, Biochem. J. 62, 160 (1956).

<sup>14</sup> A. E. BEET, Nature 175, 514 (1955).

<sup>15</sup> K. LOHMANN und L. JENDRASSIK, Biochem. Z. 178, 419 (1926).

<sup>16</sup> D. I. ARNON, Plant Physiol. 24, 1 (1949).



Wir danken der Deutschen Forschungsgemeinschaft für ihre Unterstützung.

R. THALACKER und M. BEHRENS

*Physiologisch-Chemisches Institut der Justus Liebig-Universität in Gießen, 22. Juli 1959.*

### Summary

For the isolation of chloroplasts in non-aqueous media the following organic solvents are suitable: pentane, hexane, heptane, petroleum ether and carbon tetrachloride. These solvents cause a minimum loss of lipids.

The loss of lipids and some lipid components of frozen-dried and ground shoots from *Elodea canadensis* during the isolation of chloroplasts in non-aqueous media (petroleum ether b. r. 60–80°C and petroleum ether-carbon tetrachloride mixtures) is reported.

## PRO EXPERIMENTIS

### Preparation of Subcellular Particles from Brain Tissue by a Filtration Technique<sup>1</sup>

The chemistry and physiology of subcellular particles have been extensively studied during the past several decades. These particles are usually isolated by differential centrifugation of tissue homogenates<sup>2</sup>. The present communication describes the isolation of two particulate subcellular fractions from homogenates of brain tissue by means of Millipore filters<sup>3</sup>, which are microscopic and submicroscopic sieves of cellulosic membranes with pores of precise, specified sizes. The particles in these two fractions have approximate size ranges, swelling properties and at least two biochemical properties that are similar to those of the large granules, prepared by differential centrifugation, to which the term 'mitochondria' is frequently applied.

Millipore filters have had several applications in the biological field. For example, they have been employed in the concentration of bacteria and viruses, which are in the size range of several classes of subcellular particles.

**Methods.** All operations in preparation of the subcellular fractions were carried out at 0°–4°C. 1 g of mixed grey and white matter from the cerebral hemispheres of Sprague-Dawley male rats was placed in sufficient 0.25 M sucrose solution to make the final volume 10 ml, and homogenization effected in a Dounce all glass homogenizer by 10 strokes with the loosely fitting plunger and 5 strokes with the tightly fitting plunger. The homogenate was passed successively through electrodeposited nickel screens<sup>4</sup> of approximately 40 and 20  $\mu$  pore sizes. These filtrations removed tissue debris, whole cells and many nuclei. This is essential to avoid clogging of the filters with small pore size. Next the filtrate was passed through Millipore filter SM (pore size  $5.0 \mu \pm 1.2 \mu$ )<sup>5</sup>. Nuclei, red cells, and some of the largest rod shaped mitochondria were removed at this step. The filtrate was then passed through Millipore filter SS (pore size  $3 \mu \pm 0.9 \mu$ ). This filtrate will be designated SSF. The residue on the SS filter disc was recovered by removing the disc from the filter holder, turning it upside down, replacing it in the holder and running 2 ml of 0.25 M sucrose through the

filtration apparatus, under suction. This resuspended residue is designated SSR. SSF was now put through the Millipore filter AA (pore size  $0.80 \mu, \pm 0.05 \mu$ ). The residue on the disc, designated AAR, was removed as described previously.

Suction of minus 20–26 inches mercury vacuum was used at each filtration step. 4.7 cm filter discs were employed. The XX20 047 000 Hydrosol standard filter holder manufactured by the Millipore Corporation was used for support of the filter discs. The filter discs were placed directly on the wire screen. At 0°–4°C ambient temperature, great care must be exercised in manipulation to avoid producing minute cracks in the Millipore filters, especially when they are dry. Suction must be applied gently for the same reason. Failure to observe these precautions may lead to badly contaminated fractions.

Adenosine triphosphatase and nitrogen determinations were done by methods similar to those previously reported from this laboratory<sup>6</sup>. Cytochrome *c* oxidase determinations were done spectrophotometrically by the method of COOPERSTEIN and LAZAROW<sup>7</sup>.

Phase microscopy was used in optical study of the fractions. Sizes of the particles were estimated by means of a filar micrometer. Because of Brownian movement of the particles, these estimates are very difficult to make with accuracy and should be regarded as only rough approximations.

Swelling of the particles was studied by observing changes in their size under direct microscopic observation. Solutions of sucrose with osmotic strengths lower than that of the standard 0.25 M sucrose suspension media were run beneath the cover slip and changes in particle size were noted.

For the purposes of comparison with filtration fractions SSR and AAR, the subcellular fraction that sediments after centrifugation for 15 min at  $12000 \times$  gravity – the 'mitochondrial' fraction – was prepared from rat brain homogenate according to procedures previously described<sup>6</sup>. The fraction was not washed. It will be referred to as 12G15<sup>6</sup>.

**Results.** The two fractions SSR and AAR contain spherical, comma, and rod shaped particles morphologically indistinguishable in the phase microscope from the particles seen in the 12G15 or 'mitochondrial' fraction prepared by differential centrifugation. There was not a clear cut separation of particles with respect to size in the two fractions. In SSR there was a preponderance of the larger particles from approximately 2.5 to 4.0  $\mu$  in size. However, many of the smaller particles which are the main constituents of AAR were present in SSR. Rarely, a red cell was encountered in SSR. AAR consisted largely

<sup>1</sup> We are grateful to the David Baird Grant in Neurological Research of the United Cerebral Palsy Research and Educational Foundation, Inc. and to the National Institute for Neurological Diseases and Blindness, National Institutes of Health, United States Public Health Service (Grant B-305 C), for generous support of this work.

<sup>2</sup> R. R. BENSLEY and N. L. HOERR, *Anat. Rec.* **60**, 449 (1934).

<sup>3</sup> Can be obtained from the Millipore Corporation, Bedford, Massachusetts. In the early phase of our work some filters were supplied gratis. We are also indebted to their staff for helpful advice.

<sup>4</sup> Kindly supplied gratis by the Pyramid Screen Corporation, Brookline, Massachusetts.

<sup>5</sup> Details of calibration and standardization of these filters can be obtained from the Millipore Corporation.

<sup>6</sup> W. K. JORDAN and R. MARCH, *J. Histochem. Cytochem.* **4**, 301 (1956).

<sup>7</sup> S. J. COOPERSTEIN and A. LAZAROW, *J. biol. Chem.* **189**, 665 (1951).



of smaller spheres and very thin rods ranging from *approximately* 1.0 to 2.0  $\mu$ . Some of the larger particles seen in SSR were also in AAR. Some very small particles less than 0.8  $\mu$  were also seen in AAR.

The sizes of particles in the 12G15 fraction were from 1  $\mu$  to 4  $\mu$ , which is the range reported by BRODY and BAIN<sup>8</sup> in their original description of the microscopic characteristics of this fraction. We observed some particles in this fraction which were less than 1  $\mu$ . The microscopic observations of BRODY and BAIN were done on washed 12G15 fractions, which may account for this difference.

Lowering of osmotic strength of the ambient liquid medium produced swelling in most of the particles in both filtration fractions.

Aging the filtration fractions for periods of from 1-8 h at 4°C induced crescent formation in many of the particles.

The swelling properties and crescent formation of 12G15 particles were similar to those of fractions AAR and SSR.

In the Table are summarized the results of observations on the adenosine triphosphatase and cytochrome c oxidase activities of these two fractions. For comparison, similar observations on fraction 12G15 prepared by differential centrifugation are reported. As can be seen, the specific activities of these two biochemical reactions are in the same range in all three particle groups.

The specific activity of ATPase is expressed as the micrograms of phosphorus released per h per mg of nitrogen. The specific activity of cytochrome c oxidase is expressed as the decrease in log<sub>10</sub> of the concentration of ferrocytochrome c/min/mg of nitrogen. Designations of the fractions are defined in the text. Results are the means of 4 experiments

| Fraction | ATPase | Cytochrome c Oxidase |
|----------|--------|----------------------|
| 12G15    | 3257   | 28.2                 |
| SSR      | 3536   | 19.7                 |
| AAR      | 3994   | 21.8                 |

Preparation of the particles by filtration could be effected in about half the time required by the differential centrifugation technique. The final yield was less than that obtained by centrifugation.

*Discussion.* By means of filtration, two fractions of subcellular granules can be isolated from brain homogenates that resemble in morphological, swelling, and two biochemical properties, the 'mitochondrial' fraction obtained by differential centrifugation. This technique requires less time than isolation by centrifugation, and hence the particles prepared by the filtration procedures are exposed to unnatural conditions for a shorter period before study than are those prepared in the centrifuge. In view of the well known 'aging' effect on biochemical and other properties of the particles, this may prove advantageous in certain types of investigation. For this reason, and because of the simplicity of the equipment required, the filtration technique for preparation of subcellular granules may serve as a useful alternative to the differential centrifugation procedure under some circumstances. Fractions SSR and AAR can be used singly or in combination.

With the 4.7 cm filter discs and with 10 ml of a 10% homogenate, separation of particles in respect to size is not precise, after one filtration. This is apparently the result of several factors. The larger particles doubtless pile up on the upper surface of the filter and trap the smaller particles remaining in the suspension above the filter. Also, some of the rod shaped particles probably slip through the filter sideways. We have tried repeated filtration with the 4.7 cm filters, analogous with repeated washings in the differential centrifugation procedure, and this does lead to much sharper resolution of particle size. However, with the 4.7 cm filters and with the quantity of homogenate used the reduction in yield with repeated filtrations is too great to provide preparations of practical value for most biochemical determinations.

The Millipore filters are available in sizes up to 60 cm by 130 cm. We have under construction a filter holder that will accommodate the larger filters and also will incorporate features making it more suitable for cell fractionation work than the present commercially available holders.

The initial impression we have derived from our experience with these filters is that with the equipment presently available they may have a definite, though limited, use in biochemical cytology. However, after improvement of apparatus and refinement of technique they may prove to have a wider applicability.

In addition to investigations directed toward improvement in methods for preparing the 'mitochondrial' fraction, studies on the preparation of other subcellular fractions by Millipore filters are in progress in our laboratory.

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#### Zusammenfassung

Es wird eine Technik für die Isolierung von subzellulären Partikeln in der Grössenordnung von 1-4  $\mu$  («Mitochondrien»-Fraktion) von Gehirnhomogenaten mittels Millipore-Filter beschrieben. Die Partikel zeigen morphologische Quellung und haben mindestens zwei biochemische Eigenschaften, ähnlich denjenigen von durch differentiale Zentrifugation gewonnenen «Mitochondrien»-Fraktionen. Diese Technik kann als alternative Methode zur Präparierung von subzellulären Grossteilchen-Fractionen herangezogen werden.

<sup>8</sup> T. M. BRODY and J. A. BAIN, J. biol. Chem. 195, 685 (1952).

#### CORRIGENDUM

R. JAKUES und R. MEIER: Über eine Strahlenschutz-wirkung von Apresolin und C. 5864-Su (2-Octahydro-1-azocinyl-äthyl-guanidin). Exper. vol. XVI, fasc. 2, p. 75 (1960).

Aus Versehen wurde im obigen Titel «azocinyl» gedruckt, statt «azocinyl».





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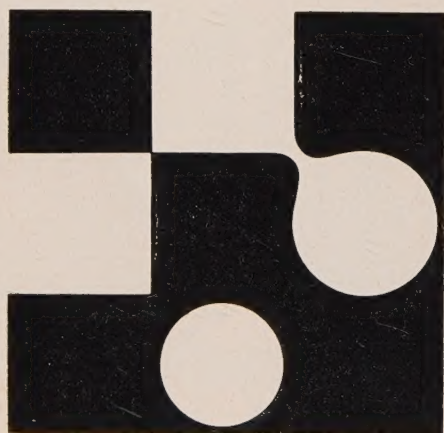
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